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| 12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited | | | 12b. DISTRIBUTION CODE | |
| 13. ABSTRACT (Maximum 200 words) This proposal investigated the potential efficacy of cultured keratinocyte allografts for victims of massive burn wounds. The results of sequential studies have shown that cultured keratinocyte (CK) allografts do induce some immune responsiveness, but not the equivalent of full thickness skin allografts. This immune responsiveness is considerably decreased in recipients who have been burned, and this decrease is relative to burn size. Studies of the mechanism of this effect have demonstrated that burn injury decreases the immunogenicity of cultured keratinocyte allografts by down-regulating the antigen expression of donor cells, as well as impairing alloantigen processing and effector cell function of the recipients. Additional findings from this research have noted that the passenger fibroblasts that persist in cultured keratinocyte grafts can also contribute to the immune responsiveness to CK grafts and should be eliminated if possible. Although CK allografts are not totally devoid of the capacity to prime animals for rejection, the impaired sensitization in burn victims suggests that this approach may indeed be useful with additional investigation and continued improvement in techniques. | | | | |
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FOREWORD

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Anthony A. Meyer 6/1/95
PI - Signature Date

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INTRODUCTION

This proposal (DAMD17-91-Z-1007) was undertaken to determine if Cultured Keratinocyte Allografts (CK Allo) are potentially useful in wound coverage after massive thermal injury, an injury suffered in a significant number of military personnel.

Autologous Cultured Keratinocytes (CK Auto) have been successfully used in our Burn Center and other institutions (1-3). However, delay in availability and varied success have caused these CK Auto to be of limited use (1,3). CK Allo could be available immediately and in essentially unlimited supply in a few centers.

CK Allo have been reported to be used with some long term success, but definitive proof of graft survival is lacking (4,5). Although keratinocyte grafts appear to be less immunogenic than skin grafts because keratinocytes do not constitutively express Class II histocompatibility antigen, their immunogenicity in CK allo transplants had not been well studied (6,7).

This proposal (Log No 88 175001-LAIR) investigated the immunogenicity of CK Allo to determine if this technique may be useful in treating military and civilian massive burn injuries. The studies done have addressed these hypotheses and gone well beyond them in several areas. The results of these studies will be summarized in the Body of this report.

This work represents a considerable amount of effort by all parties concerned. Specifically, I would like to acknowledge the contributions of Dr. Bruce Cairns and Dr. Scott Hultman, and Ms. Suzan deSerres, the research analyst who helped coordinate and provide continuity for the studies.

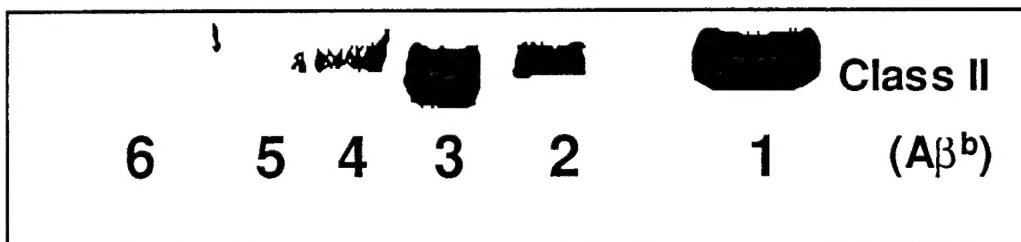
BODY OF REPORT

The results of the studies will be summarized under the major headings of keratinocyte biology, host response to cultured keratinocyte allografts, the effect of burn injury on host response to cultured keratinocyte allografts, and the role of passenger fibroblasts in cultured keratinocyte grafts. Following review of these four areas, the individual hypotheses from the original proposal will be restated with brief conclusions to each hypothesis.

I. KERATINOCYTE BIOLOGY

- A. Cultured keratinocyte grafts do not express Class II histocompatibility antigen in vitro in normal conditions. With the introduction of interferon gamma or certain other cytokines, keratinocytes can be induced to express Class II histocompatibility antigen. These findings were described previously and reconfirmed in our laboratory.
- B. After transplantation, cultured keratinocyte allografts do express Class II histocompatibility antigen. This occurs without application or injection of exogenous interferon gamma. This expression of Class II histocompatibility antigens in cultured keratinocyte grafts does decrease over time with a peak at approximately two to three days after grafting. These findings are demonstrated by Figure 1 which comes from our work published in Transplantation (8). The original figure can be found in Figure 4 in the Appendix article entitled "Cultured mouse keratinocyte allografts prime for accelerated second set rejection and enhanced cytotoxic lymphocyte response".

Figure 1. H-2^b
MHC class II
antigen expression in
B6 CK allografts.
Western immunoblot



of biopsies obtained from flank of graft recipients using monoclonal antibody specific for B6 class II antigen; 1: positive control specimen for MHC class II antigen; 2: two days post grafting; 3: three days post grafting; 4: five days post grafting; 5: seven days post grafting; and 6: B6 CK in vitro.

- C. Cultured keratinocyte allografts transplanted onto burn animals express less histocompatibility antigen than do identical CK Allo transplanted to normal animals (9). Evidence for these studies is shown in Figure 2. The reduction in Class II antigen expression is associated with the burn size and depth. The levels of interferon-gamma in the wound are shown in Figure 3 which shows some proportional changes in interferon-gamma levels with Class II antigen expression. The relative increase in wound interferon-gamma in the 20% burns despite a decrease in Class II antigen production may be related to the involvement of TNF alpha and other cytokines in controlling Class II antigen expression after burn injury.

Figure 2. The effect of burn size on MHC Class II antigen expression in CK allografts, three days after excision and grafting. Immunoblots were scanned with video densitometry to quantify alloantigen expression, which is depicted as mean adjusted pixel density. Error bars represent standard error of the mean. Both 20% FT and 40% FT burn injuries significantly inhibited Class II alloantigen expression. * $p < 0.05$ vs 0%; ** $p < 0.001$ vs 0%.

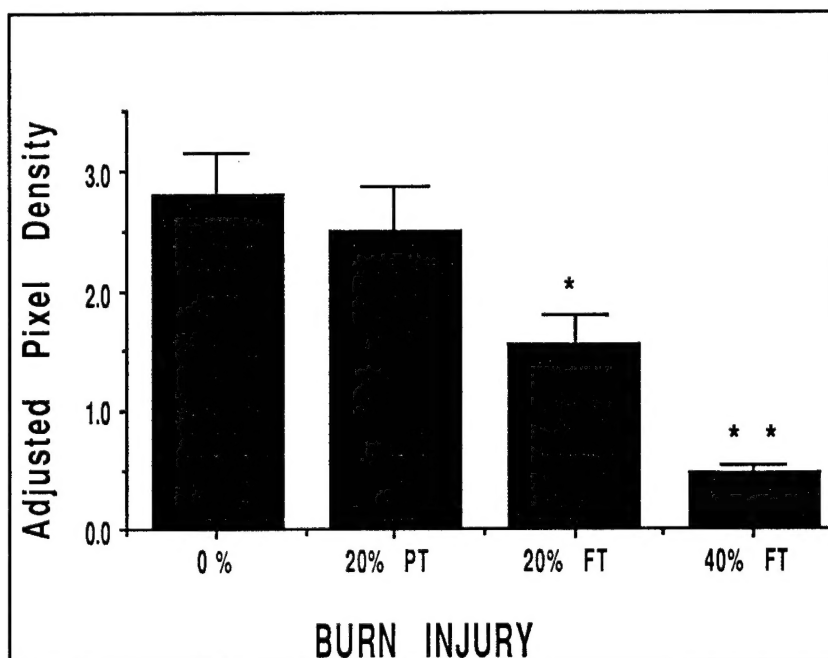
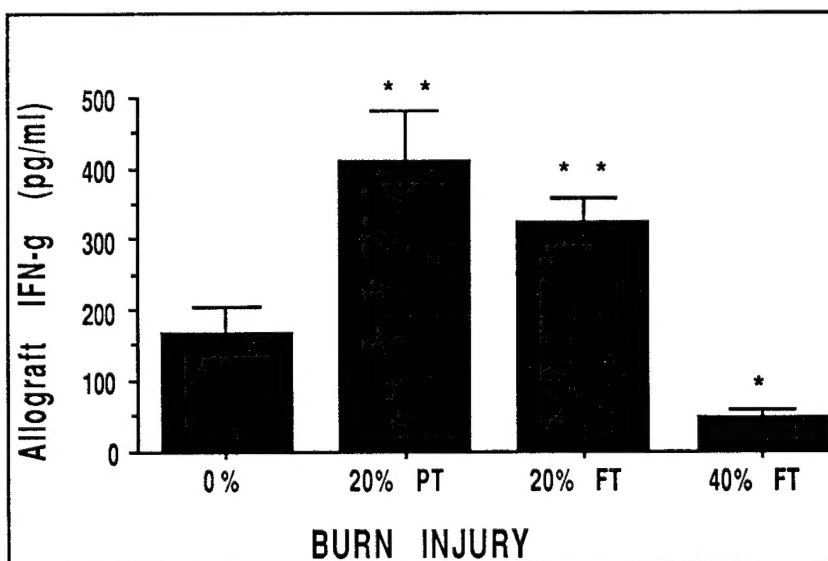


Figure 3. The effect of burn size on IFN- γ levels in grafted wounds. Both 20% PT and 20% FT injuries significantly increased wound IFN- γ , whereas 40% FT burn decreased wound IFN- γ . * $p < 0.05$ vs 0%, 20% PT, 20% FT; ** $p < 0.01$ vs 0%, 40% FT.



II. HOST RESPONSE TO CULTURED KERATINOCYTE ALLOGRAFTS

- A. Initial studies in this proposal address the immune response to cultured keratinocyte allografts in a mouse model. Mixed lymphocyte response, a measure of Class II antigen response, and cytotoxic antibody, a measure of Class I antigen response, were found not to be present in mice who had received cultured keratinocyte allografts as compared to those receiving full thickness allografts (10). These data are demonstrated in Figures 4 and 5. This initially led us to believe that keratinocytes may be immunologically privileged and would not elicit an immune response to transplanted allogeneic cells.

Figure 4. MLR results for each group combined over all experimental days; * $p < 0.001$ by MANOVA.

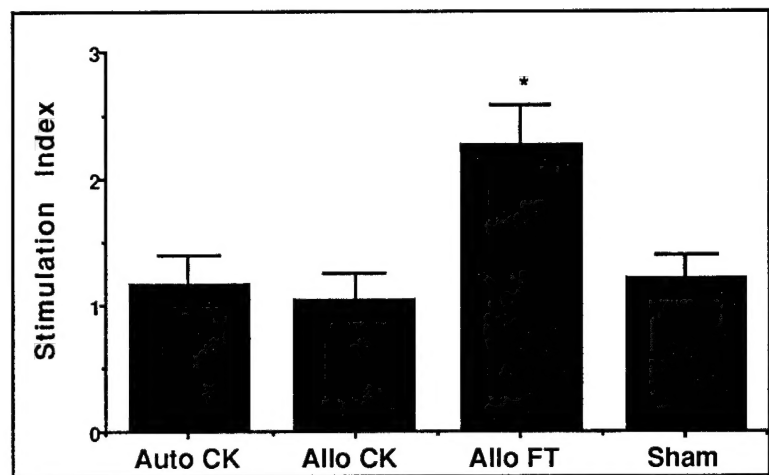
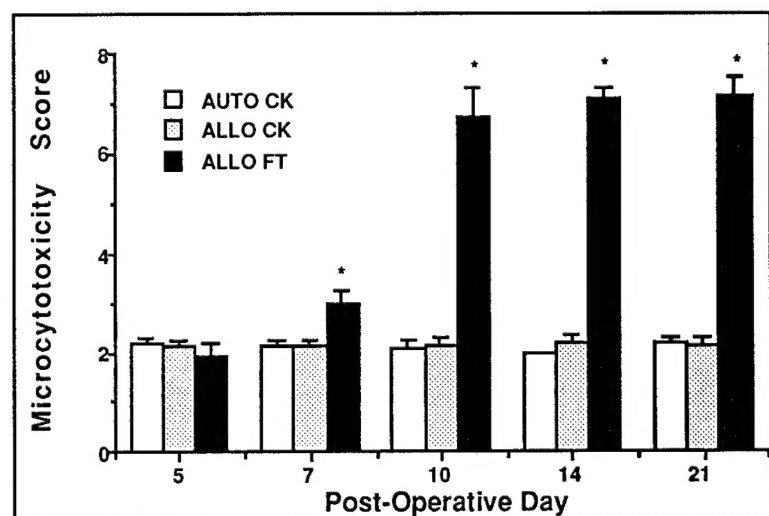


Figure 5. Cytotoxic antibody levels in undiluted serum for each group as a function of post-operative day. NIH score ≥ 4 is positive and represents 50% cell killing; * $p < 0.05$ by MANOVA.



B. However, after evaluating cultured keratinocyte allografts by the more sensitive and clinically relevant *in vivo* method of accelerated second set rejection, we found that CK allografts induced an immune response in recipient mice for second set rejection equal to animals sensitized with full thickness allograft. These data are shown in Figure 6 and were published in Transplantation (8). In order to come up with a cellular mechanism to explain the priming for enhanced second-set rejection, we investigated the presence of cytotoxic lymphocytes in animals primed with CK allo. This study showed that CK allografts induced a significant cytotoxic lymphocyte response, although not as strong as full thickness allografts, considerably more than the negative control of cultured keratinocyte autografts shown in Figure 7.

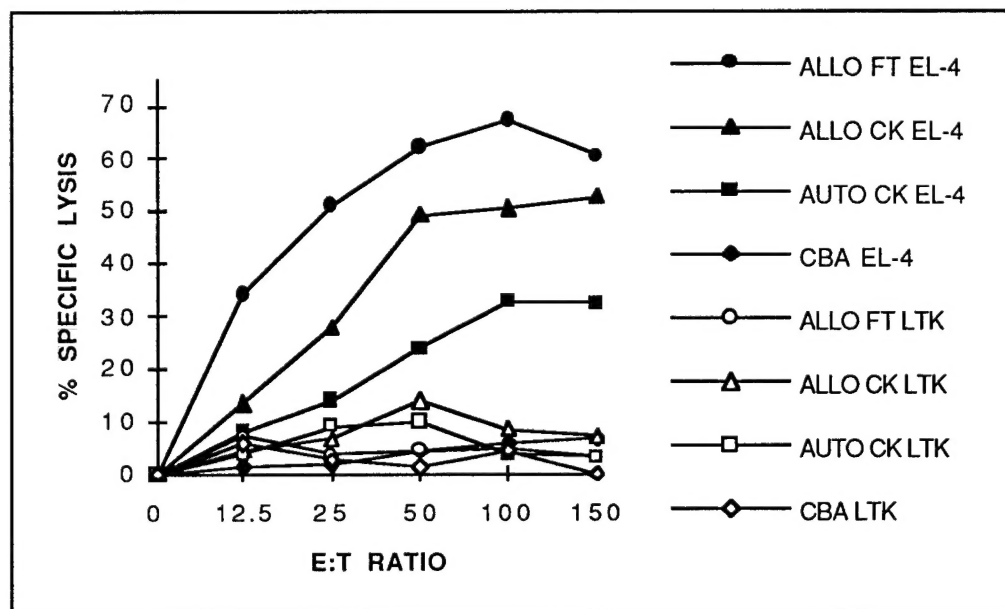
Figure 6. Second Set

Graft Survival. ^a Number days graft survived is bold; "()" is number of grafts rejected at that day; ^b MST, median survival

| Flank Graft | Tail allograft survival(days) ^a | MST (days) ^b |
|----------------|--|-------------------------|
| ALLO FT (n=21) | 7, 8(6) , 9(7), 10(6), 11 | 9 |
| ALLO CK (n=22) | 8(2), 9(9), 10(8), 11, 13(2) | 9 |
| AUTO CK (n=17) | 11(2), 12(3), 13(7), 14(4), 16 | |
| CBA (n=11) | 9, 11, 13(6), 14(3) | 13 |

time. Allo CK and Allo FT vs Auto CK and CBA $\chi^2 > 18$, $P < 0.01$.

Figure 7. Recipient CTL after 4 days of *in vitro* allostimulation



III. EFFECT OF BURN INJURY ON HOST RESPONSE TO CK ALLO

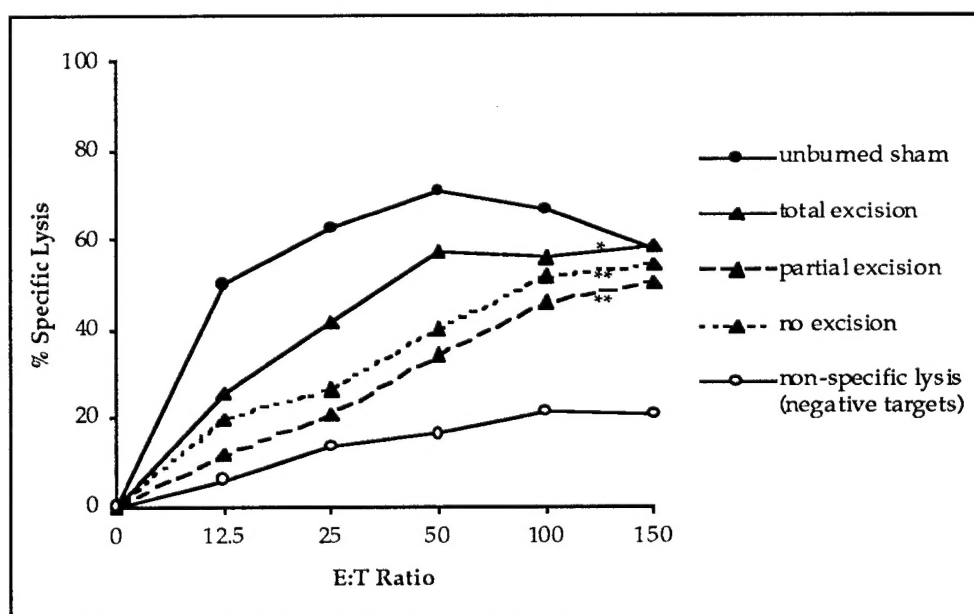
A. Studies in our model were done to confirm that burn injury limits both primary and second set responsiveness to tail grafts. Furthermore, this effect is dependent upon burn size (11). These data are shown in Figure 8, which shows the effect of burn injury on these parameters.

| | PRIMED Primary flank graft: allograft | | | UNPRIMED Primary flank graft: autograft | | |
|------------|--|----------|----------|--|----------|----------|
| | 0% burn | 20% burn | 40% burn | 0% burn | 20% burn | 40% burn |
| MST, days | 9 | 10 * | 12.5 * | 13 | 14 * | 15 * |
| MST, range | 7-11 | 8-12 | 10-16 | 10-15 | 12-17 | 13-18 |
| N | 18 | 16 | 16 | 15 | 17 | 13 |

Figure 8. The effect of burn injury and priming on the survival of second set tail allografts. Mice receiving primary flank allografts are defined as PRIMED, whereas mice receiving primary flank autografts are defined as UNPRIMED. MST=median survival time of secondary tail allografts. Both 20% and 40% burn injury impaired host responsiveness and significantly prolonged second-set rejection, compared to 0% controls. * $p < 0.05$ vs 0% burn.

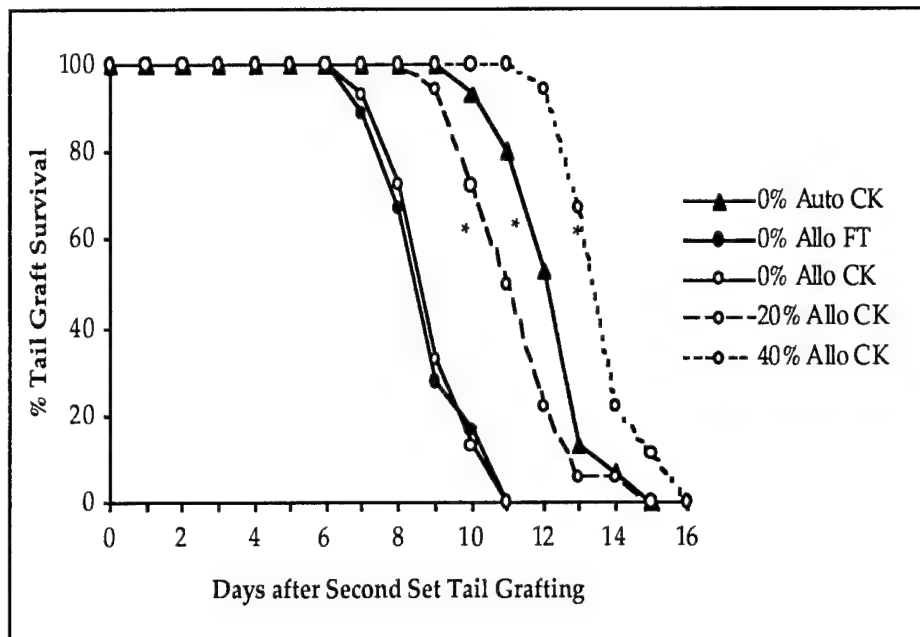
B. The effect of burn injury to impair cytotoxic lymphocyte response was shown to be partially abrogated by excision and grafting of the full thickness burn wound (12). The data in Figure 9 show these effects.

Figure 9. The effect of burn wound excision on CTL function, 4 days after in vitro stimulation. * $p < 0.01$ vs unburned sham, partial excision, no excision; ** $p < 0.01$ vs unburned sham, total excision.



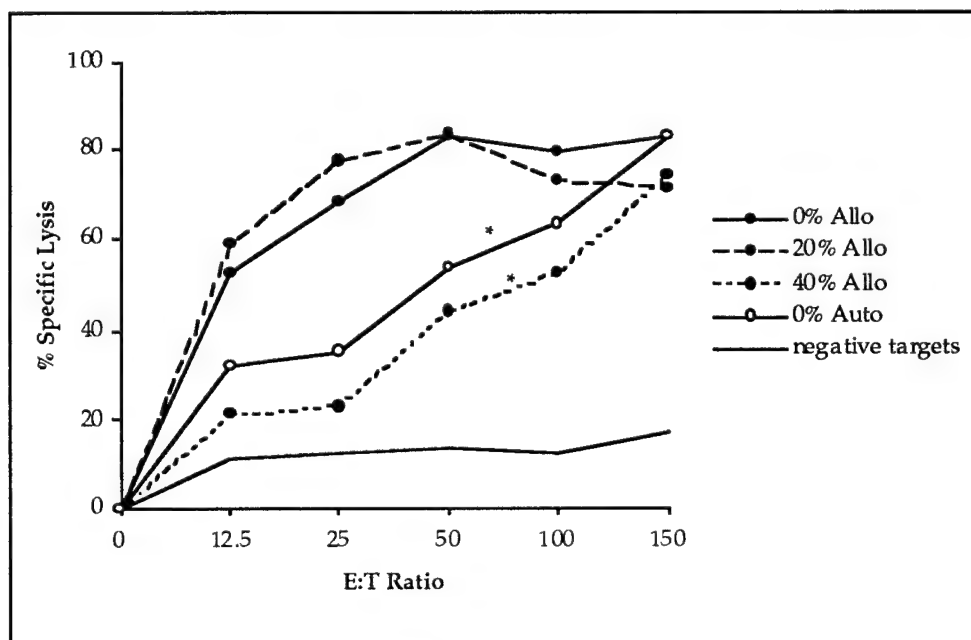
- C. Burn injury, however, has a greater effect on the impairment of CK allograft sensitization than it does on full thickness sensitization. When compared to rejection response in unprimed, unburned animals, a 40% full thickness body surface area burn eliminates the effects of second set rejection to a level equal to that of unburned animals (13). These data are shown in Figure 10.

Figure 10. Survival curves of secondary tail allografts, depicting the effect of burn injury on second set rejection. Both Allo FT and Allo CK primed the unburned host with equal efficacy. Burn injury significantly inhibited sensitization by Allo CK, as evidenced by prolonged survival of second set tail allografts. * $p < 0.05$ vs 0% Allo FT and 0% Allo CK.



- D. The mechanism by which burn injury impedes second set rejection and cytotoxic lymphocyte function involves both antigen processing as well as effective mechanisms of allograft cell destruction. These data, shown in Figure 11, were recently presented at the 1995 American Burn Association Meeting for which Dr. Hultman received the Moyer Award as the best paper.

Figure 11. The effect of burn injury on CTL alloreactivity, 5 days after in vitro stimulation. * $p < 0.05$ vs 0% Allo and 20% Allo.



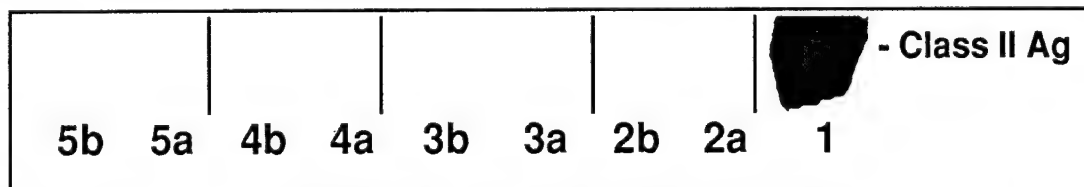
IV. PASSENGER FIBROBLASTS IN CULTURED KERATINOCYTE GRAFTS

- A. As a part of our initial studies, we found histocompatibility antigens in cultured keratinocytes that could not be explained by the strain of the animal used as a keratinocyte donor. Further investigation showed that the Swiss-albino 3T3 fibroblasts treated with mitomycin-C to prevent growth and used as a feeder layer to grow the keratinocytes, did persist in CK grafts and did express Class II histocompatibility antigen (14). These data are shown in Figure 12, which demonstrates fluorescence activated cell sorter numbers identifying a 0-3% population of passenger fibroblasts in mature third passage keratinocyte grafts. Figure 13 shows that these cells express Class II histocompatibility antigens when stimulated with interferon-gamma or grafted onto animals. The original of Figure 13 is in the Appendix article entitled "Xenogeneic fibroblasts persist in human cultured epidermal autografts: a possible mechanism of late graft loss", Figure 4.

Figure 12. Fibroblast Persistence by Western Immunoblot/ Blots were probed with KL295, an anti-murine MHC class II antibody, designated as positive (+), negative (-), or not done (ND).

| Patient | Culture Passage | | |
|----------|-----------------|-----------|----------|
| | Primary | Secondary | Tertiary |
| A | + | + | - |
| B | + | - | - |
| C | + | + | + |
| D | + | + | + |
| E | + | + | + |
| F | + | + | + |
| G | + | - | - |
| H | + | + | + |
| I | + | + | ND |
| K | + | + | + |

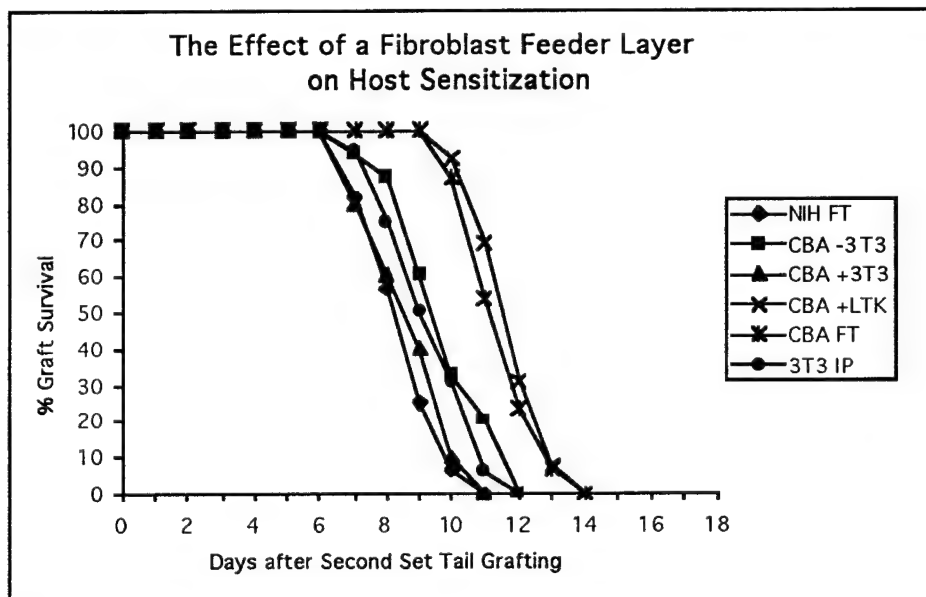
Figure 13. Western immunoblot detection of mouse MHC class II antigen (Ag) in human CEA. 1, positive control for MHC class II Ag; 2a, Primary CEA on LTK⁻ feeder layer (FL); 2b, Primary CEA on LTK⁻ FL treated with interferon-gamma (IFN-g); 3a Primary CEA on 3T3 FL; 3b Primary CEA on 3T3 FL treated IFN-g; 4a, Secondary CEA on LTK⁻ FL; 4b, Secondary CEA on LTK⁻ FL treated with IFN-g; 5a Secondary CEA on 3T3 FL; 5b Secondary CEA on 3T3 FL treated IFN-g. MHC class II Ag only present in 3b and 5b: CEA on 3T3 FL treated with IFN-g.



- B. Recent studies to be presented at the American Association for the Surgery of Trauma meetings in September 1995 have shown that these passenger cells are immunologically active and prime recipients for accelerated second set rejection of skin specific to the fibroblast donor and not to the keratinocyte (15). These data are shown in Figure 14 and summarized in Figure 15.

Figure 14. The effect of a fibroblast feeder layer on host sensitization. CBA mice (n=85) were primed with NIH FT flank grafts (NIH full-thickness skin; positive control), CBA-3T3 flank grafts (CBA cultured keratinocytes grown on a 3T3 feeder layer that was removed; experimental group), CBA+3T3 flank grafts (CBA cultured

keratinocytes grown on a 3T3 feeder layer that was not removed; experimental group), CBA+LTK flank grafts (CBA cultured keratinocytes grown on an LTK feeder layer that was not removed; negative control), CBA FT flank grafts (CBA



full-thickness skin; negative control), and 3T3 IP fibroblasts (3T3 fibroblasts injected intraperitoneally; experimental group). Three weeks after priming, mice were challenged with tail allografts (NIH FT). Survival curves of second-set tail grafts are depicted. Mice primed with NIH FT, 3T3 IP, CBA+3T3, and CBA-3T3 demonstrated accelerated second-set rejection ($p<0.05$). These findings suggest that the foreign fibroblast feeder layer used to cultivate cultured keratinocytes persists until grafting and remains immunogenic.

| Primary Flank Graft | NIH allo (+) control | CBA+3T3 allogeneic FB | CBA+LTK syngeneic FB | CBA auto (-) control |
|--------------------------|----------------------|-----------------------|----------------------|----------------------|
| Secondary Graft Survival | 9 days* | 10 days* | 12 days | 12 days |

Figure 15. Allogeneic fibroblasts persisted after grafting but decreased over time, as determined by alloantigen expression on Western immunoblots. Accelerated tail graft rejection occurred in hosts primed by NIH allografts ($*p<0.05$), as well as by CEAs grown with an allogeneic (3T3) but not syngeneic (LTK) feeder layer ($*p<0.05$).

These findings show coordinated, sequential scientific investigation into the biology of cultured keratinocyte grafts as well as the host responsiveness to them, in normal or burned animals. This significant production of results has led to nine publications that are either in print or accepted for publication. These publications are listed and included in the Appendix, along with the titles of thirteen presentations given at national scientific meetings, and two presentations to be given later in the year. An additional manuscript is being prepared for presentation and several other experiments are in progress.

The hypotheses in the original proposal are re-stated and the results summarized to address each hypothesis in the following section.

HYPOTHESES

A. Cultured keratinocyte allografts do not routinely express Class II histocompatibility antigens and have been successfully allografted.

These studies were confirmed in our laboratory and although CK allografts have been successfully performed in our lab, they do not have proven longevity.

B. Cultured keratinocyte allografts can be induced to express Class II histocompatibility antigens by interferon-gamma.

We have demonstrated that CK allografts express Class II histocompatibility antigen with interferon-gamma in vitro, and express this antigen in vivo even without supplemental interferon-gamma.

C. Delayed expression of Class II histocompatibility antigens by cultured allograft keratinocytes will lead to late graft rejection.

We do not have evidence that the primary CK allografts have any difference in survival compared to CK autografts. However, the CK allografts induce less of a cytotoxic T-cell response compared to full thickness allografts.

D. Keratinocyte allograft recipients with immunosuppression secondary to burn injury will reject allografts after the expression of Class II histocompatibility antigens.

Burn injury does produce immunosuppression which leads to impaired Class II antigen expression, diminished CTL response and impaired priming for second set rejection. The effect of burn injury on primary CK allograft survival is still unclear.

E. Keratinocyte allografts will prime the recipient for rejection of subsequent identical allografts only after they are induced to express Class II histocompatibility antigen.

Since the CK allografts express Class II histocompatibility antigen upon grafting, they prime the animals as demonstrated by enhanced second set rejection.

CONCLUSIONS

Conclusions based on the results described in the preceding pages are the following:

1. CK allografts can be successfully transplanted onto recipient animals in this mouse model.
2. These allografts do sensitize the animals as measured by priming for second set rejection and CTL function, but not as measured by mixed lymphocyte response and cytotoxic antibody.
3. Burn injury significantly impairs the ability of the host to be sensitized to allografts and there is a greater effect on cultured keratinocyte allografts than full thickness allografts.
4. Additional culture techniques to eliminate passenger fibroblasts from cultured keratinocyte grafts or to substitute non-immunogenic cells must be developed to avoid this possible source of graft rejection.
5. Although cultured keratinocyte allografts have not been proven to induce tolerance, their diminished immunogenicity in a burn model suggest that they may indeed still be useful in grafting of victims of massive burns.
6. These studies have led to a considerably increased understanding of the knowledge of keratinocyte allografts and their immune responsiveness, as well as possible causes of limited success in keratinocyte autografts. The future studies outlined in the renewal application proceed with these investigations and may potentially lead to the development of a keratinocyte cell line that could serve as a universal donor by deletion of genes that sensitize recipients for graft rejection.

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10. Cairns BA, deSerres S, Kilpatrick K, Frelinger JA, Meyer AA. Cultured keratinocyte allografts fail to induce sensitization in vivo. *Surgery*, 114(2):416-422, 1993.
11. Hultman CS, Cairns BA, deSerres S, Frelinger JA, Meyer AA. Burn injury impairs second set rejection and CTL reactivity in mice primed by cultured keratinocyte allografts. *Transplantation* (in press).
12. Hultman CS, Cairns BA, deSerres S, Frelinger JA, Meyer AA. Early, complete burn wound excision partially restores cytotoxic T lymphocyte function. *Surgery* (in press).
13. Hultman CS, Cairns BA, Yamamoto H, deSerres S, Frelinger JA, Meyer AA. The effect of burn injury on allograft rejection, alloantigen processing, and cytotoxic T lymphocyte sensitization. *The Journal of Burn Care and Rehabilitation* (in press).
14. Cairns BA, deSerres S, Brady LA, Hultman CS, Meyer AA. Xenogeneic mouse fibroblasts persist in human cultured epidermal grafts: a possible mechanism of graft loss. *J Trauma* (in press).
15. Hultman CS, Brinson GM, Siltharm S, Cairns BA, deSerres S, Meyer AA. Allogeneic fibroblasts used to culture epidermal autografts persist in vivo and sensitize the graft recipient for accelerated second set rejection. *Journal of Trauma*. In preparation.

APPENDIX

PUBLICATIONS:

- Cairns BA, deSerres S, Kilpatrick K, Frelinger JA, Meyer AA. Cultured keratinocyte allografts fail to induce sensitization in vivo. *Surgery*, 114(2):416-422, 1993.
- Cairns BA, deSerres S, Matsui M, Frelinger JA, Meyer AA. Cultured mouse keratinocyte allografts prime for accelerated second set rejection and enhanced cytotoxic lymphocyte response. *Transplantation*, 58:67-72, 1994.
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- Cairns BA, deSerres S, Brady LA, Hultman CS, Meyer AA. Xenogeneic mouse fibroblasts persist in human cultured epidermal grafts: a possible mechanism of graft loss. *J Trauma* (in press).
- Hultman CS, Cairns BA, deSerres S, Frelinger JA, Meyer AA. Burn injury impairs second set rejection and CTL reactivity in mice primed by cultured keratinocyte allografts. *Transplantation* (in press).
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- Hultman CS, Brinson GM, Siltharm S, Cairns BA, deSerres S, Meyer AA. Allogeneic fibroblasts used to culture epidermal autografts persist in vivo and sensitize the graft recipient for accelerated second set rejection. *Journal of Trauma*. In preparation.

PRESENTATIONS:

Cairns BA; deSerres S; Kilpatrick K; Frelinger JA; Meyer, AA. "Cultured keratinocyte allografts fail to induce sensitization in vivo". Presented at the 45th Annual Meeting of the Society of University Surgeons, Seattle, WA. February 13, 1993.

Cairns BA; deSerres S; Brady LA; Meyer AA. "Fibroblasts from feeder layer persist in cultured keratinocyte grafts". Presented at the 25th Annual Meeting of the American Burn Association, Cincinnati, OH. March 25, 1993.

Cairns BA; deSerres S; Meyer AA. "Effects of wound coverage on the systemic immune response". Presented at the 25th Annual Meeting of the American Burn Association, Cincinnati, OH. March 25, 1993.

Cairns BA. "Immunogenicity of cultured keratinocyte skin grafts". Surgery Grand Rounds, University of North Carolina. April 14, 1993.

Cairns BA; deSerres S; Frelinger JA; Meyer AA. "Cultured keratinocyte allografts express MHC class II antigens and induce accelerated second-set rejection". Presented at the 27th Annual Meeting of the Association of Academic Surgeons, Hershey, PA. November 13, 1993.

Hultman CS, Cairns BA, Brady LA, deSerres S, Meyer AA. "Minor burn injury does not inhibit major histocompatibility class II antigen expression in cultured keratinocyte allografts". Presented at the 26th Annual Meeting of the American Burn Association, Orlando, FL. April 22, 1994.

Cairns BA, deSerres S, Brady LA, Hultman CS, Meyer AA. "Viable, immunologically active, mouse 3T3 fibroblasts persist in human cultured keratinocyte autografts used in burn wound coverage". Presented at the 54th Annual Meeting of the American Association for the Surgery of Trauma, San Diego, CA. September 29, 1994.

Hultman CS, Cairns BA, deSerres S, Brady LA, Meyer AA. "Burn injury selectively impairs host sensitization to cultured keratinocyte allografts". Presented at the Surgical Forum, 80th Clinical Congress of the American College of Surgeons, Chicago, IL. October 12, 1994.

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Hultman CS, Napolitano L, Cairns BA, deSerres S, Meyer AA. "The relationship between interferon- γ and keratinocyte alloantigen expression after burn injury". Presented at the 115th Annual Meeting of the American Surgical Association, Chicago, IL. April 7, 1995.

Hultman CS, Cairns BA, deSerres S, Meyer AA. "The effect of burn size on alloantigen recognition, processing, and priming". Presented at the 27th Annual Meeting of the American Burn Association, Albuquerque, NM. April 21, 1995.

Hultman CS, Cairns BA, Yamamoto H, deSerres S, Meyer AA. "Burn injury impairs the immunologic memory of cytotoxic T lymphocytes". Presented at the 27th Annual Meeting of the American Burn Association, Albuquerque, NM. April 21, 1995.

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Hultman CS, Brinson GM, Siltharm S, Cairns BA, deSerres S, and Meyer AA. "Allogeneic fibroblasts used to culture epidermal autografts persist in vivo and sensitize the graft recipient for accelerated second set rejection". To be presented at the 55th Annual Meeting of the American Association for the Surgery of Trauma, Halifax, Nova Scotia. September 27-30, 1995.

Hultman CS, Napolitano L, Campbell C, deSerres S, and Meyer AA. "The effect of thermal injury on local wound production of interferon- γ , tumor necrosis factor- α , and interleukin-10". To be presented at the Surgical Forum, Section on Plastic Surgery/Wound Healing, 81st Clinical Congress American College of Surgeons, New Orleans, LA. October 25, 1995.

SUMMARY OF MEETINGS AT WHICH RESULTS WERE PRESENTED:

Clinical Congress of the American College of Surgeons: 1994, 1995

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Society of University Surgeons: 1993, 1995

American Surgical Association: 1995

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American Association for the Surgery of Trauma: 1994, 1995

Association of Academic Surgeons: 1993

Cultured keratinocyte allografts fail to induce sensitization in vivo

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Background. The use of cultured keratinocyte (CK) allografts for burn wounds offers a potentially unlimited supply of skin. It is unknown, however, whether CK allografts induce rejection in vivo. This study investigated the induction of immune responsiveness to CK allografts as measured by mixed lymphocyte response and serum cytotoxic antibody.

Methods. Female CBA mice ($n = 160$) were randomized to four equal groups, each receiving a 3 cm² flank graft of autologous CBA CK (Auto CK), allogeneic C57BL/6 CK (Allo CK), C57BL/6 full thickness skin (Allo FT), or Sham. Graft take was assessed by gross and histologic examinations. Unidirectional mixed lymphocyte response was measured with graft recipient and donor splenocytes by use of tritiated thymidine uptake. Stimulation indexes were calculated. Serum cytotoxic antibody was measured by coculturing graft recipient serum with donor splenocytes and rabbit complement and assessing resultant cell killing.

Results. Overall graft take was 50% for Allo CK and 74% for Auto CK. Allo FT, but not Allo CK, were associated with significantly increased stimulation indexes compared with Auto CK and Sham ($p < 0.01$). Allo FT, but not Allo CK, resulted in elevated titers of alloantibody, reaching significant levels 10 days after grafting ($p < 0.05$).

Conclusions. CK allografts do not result in increased in vitro T cell responses or enhanced alloantibody formation, indicating that sensitization to major histocompatibility antigens by CK does not occur. These data suggest that CK allografts may provide a possible source of grafts for victims of large burn wounds. (SURGERY 1993;114:416-22.)

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RAPID AND EFFECTIVE COVERAGE of burn wounds is an important determinant of survival after major thermal injury. However, many patients lack enough donor sites to completely cover the burn wound with autologous split-thickness skin grafts. Cultured keratinocyte (CK) autografts were first reported as a permanent wound cover in 1981,¹ but the 2 to 3 weeks required to produce CK autografts have substantially limited their widespread use.² CK allografts, however, could provide immediate, unlimited amounts of material for permanent wound coverage, if they were not susceptible to rejection.

In 1983, soon after the discovery that human CK did not express major histocompatibility (MHC) class II antigens,³ the first report of successful grafting with human allogeneic CK was published.⁴ Multiple reports have been published in human studies and in animal studies that either support or refute these initial findings, and 10 years later, the immunologic response to CK allografts remains poorly defined. The purpose of our study was to determine whether CK allografts induced in vivo sensitization to MHC class I and class II antigens, as measured by mixed lymphocyte response (MLR) and serum cytotoxic antibody.

MATERIAL AND METHODS

Animals and study design. Mice used were female, 15 to 20 gm, CBA/J (CBA) (Harlan Sprague Dawley, Inc., Indianapolis, Ind.) and C57BL/6 (B6) (Charles Rivers Laboratories, Wilmington, Mass.). All animal protocols were approved by the University of North Carolina Committee on Animal Research, in accordance with National Institutes of Health (NIH) guidelines. Four groups of 40 CBA recipient mice were randomized to receive CBA CK (Auto CK), B6 CK (Allo CK), B6 full thickness skin (Allo FT), or sham surgery

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(Sham). Graft assessment and tissue harvesting for five animals in each group were performed at postoperative days 2, 3, 4, 5, 7, 10, 14, and 21.

Culture procedure. Keratinocyte cultures were performed in a method previously described for human CK,⁵ with minor modifications. Briefly, mice were killed and tail skins were harvested. Skin was finely minced and a single cell suspension was obtained by trypsinization. Keratinocytes were cultured with growth-arrested 3T3 fibroblasts at 37° C, 5% CO₂. One week after plating, fibroblasts were removed by differential trypsinization and keratinocytes were grown to confluence (about 14 to 17 days).

Skin grafting. After reaching confluence, CK grafts were separated from tissue culture plates with dispase (Boehringer Mannheim Corp., Indianapolis, Ind.), placed on petrolatum gauze, basal side up, and stored at 37° C until ready for grafting. Recipient mice were anesthetized with methoxyflurane vapor (Metofane; Pittman-Moore, Chicago, Ill.). The torso was circumferentially shaved and a 3 cm² area of skin on the left flank was excised. The gauze-backed CK graft was placed basal side down on the flank wound and tucked under the surrounding wound edge. The wound was covered with a primary wound dressing (Vigilon; C. R. Bond, Inc., Berkeley Heights, N.J.), petrolatum jelly, and a stretch fabric bandage that was secured with skin staples. Sham and Allo FT mice underwent identical surgical procedures, but Sham received no graft and Allo FT received B6 full-thickness flank skin grafts.

Graft assessment. Mice were anesthetized, bled by puncture of the orbital plexus, and then killed. Serum was separated and stored at -80° C. The wound was grossly assessed for graft presence and photographed. A 3 mm punch biopsy specimen of each wound was obtained and stored in 10% neutral buffered formalin (Fisher Scientific, Atlanta, Ga.). Specimens were stained with hematoxylin and eosin and examined microscopically for the presence of epidermal elements.

MLR. Unidirectional MLR was performed as previously published.⁶ On postoperative days 3, 5, 7, 10, 14, and 21, after anesthesia, blood sampling, death, and wound biopsy, spleens were harvested and splenocytes were isolated. Cell concentrations were adjusted to 5×10^6 cells/ml. B6 splenocytes treated with mitomycin C (Sigma Chemical Company, St. Louis, Mo.), 25 µg/ml at 37° C for 20 minutes, served as stimulators, recipient CBA splenocytes served as responders, and CBA splenocytes from untreated animals served as controls. Responder and control cells were added to individual wells of a sterile, 96-well, flat-bottom tissue culture plate (NUNC Inc., Naperville, Ill.). Untreated CBA splenocytes or mitomycin C-treated B6 splenocytes were then added to the wells, with five replicates of each control and experimental culture. Cultures were

incubated at 37° C, 5% CO₂. After 48 hours, 1 µCi tritiated methyl thymidine (ICN Biomedicals Inc., Irvine, Calif.) was added to each well and cultures were incubated for an additional 24 hours. Cells were harvested with a semiautomatic cell harvester (Skatron Inc., Sterling, Va.) onto solid scintillant filters (Xtalscint; Beckman Instruments, Columbia, Md.) and counted on a Beckman LS6000TA scintillation counter (Beckman Instruments). Stimulation index (SI) was calculated as the mean counts per minute experimental/counts per minute control for each animal. The 3 day SI for each animal and each experimental condition were computed and compared by multivariate analysis of variance (MANOVA), with statistical significance defined as $p < 0.05$.

Microcytotoxicity test. Alloantibody was detected with a modification of the complement dependent microcytotoxicity assay reported by others.⁷ B6 splenocytes were isolated on a Ficoll gradient and suspended in medium (Cedarlane; Accurate Chemical & Scientific Corp., Westbury, N.Y.) at a concentration of 2.5×10^6 cells/ml. Cells were plated on 60-well tissue typing plates with CBA graft recipient serum and rabbit complement. Plates were incubated at 37° C for 45 minutes and stained with 0.1% nigrosin. Percent lysis was determined by nigrosin uptake by nonviable cells. Assays were subjectively scored for percent cell killing, with a standardized NIH scoring system. The score ranges from 1 to 8 and is based on a continuum of cell killing. A score of 4 represents 50% cell killing and is considered a positive result. Serial dilutions of each experimental serum were performed until less than 50% cell killing was observed. Results were compared by MANOVA, with statistical significance defined as $p < 0.05$.

RESULTS

Graft assessment. Graft take for each group was assessed by visual and histologic examinations. There were no immediate postoperative deaths, but 10 to 15 days after grafting, two Auto CK mice and one Sham mouse died before graft assessment and tissue harvesting and were not included in the analysis. The graft take results are summarized in Table I. Successful graft take, as defined by histologic examination or by visual inspection, was 100% for Allo FT, 73.7% for Auto CK, and 50% for Allo CK. The best graft take for all groups was observed in the first 10 days after grafting and decreased as wound contraction occurred. The accuracy of graft take was difficult to determine because neither histologic nor gross examinations were reliable indicators. Of 33 CK grafts with visual evidence of graft take, 45% (15 of 33) had no epidermal elements on microscopic examination of biopsy specimens. Moreover, 45% (15 of 33) of wounds with histologic evidence of graft

Table I. Results of graft take for each group, expressed as percentage

| | Gross examination | Histologic examination | Gross or histologic examination |
|------------------------|-------------------|------------------------|------------------------------------|
| All postgraft days | | | |
| Auto CK | 55.2 (21 of 38) | 50.0 (19 of 38) | 73.7 (28 of 38) |
| Allo CK | 30.0 (12 of 40) | 35.0 (14 of 40) | 50.0 (20 of 40) |
| Postgraft days 2 to 10 | | | |
| Auto CK | 60.0 (18 of 30) | 56.7 (17 of 30) | 83.3 (25 of 30) |
| Allo CK | 33.3 (10 of 30) | 43.3 (13 of 30) | 60.0 (18 of 30) |

Two Auto CK animals that died 2 weeks after grafting were not included in the analysis.

Table II. Results of unidirectional MLR

| Postgraft day | Groups | | | | |
|------------------|-------------|-------------|------------|-------------|------------|
| | Auto CK | Allo CK | Allo FT | Sham | Control |
| 3 | 2.9 ± 0.95 | 2.7 ± 0.62 | 2.7 ± 0.83 | 2.01 ± 0.51 | 3.8 ± 1.2 |
| 5* | 0.67 ± 0.19 | 0.56 ± 0.18 | 1.9 ± 0.70 | 0.71 ± 0.32 | 2.4 ± 0.67 |
| 7* | 0.75 ± 0.22 | 0.86 ± 0.29 | 2.8 ± 0.95 | 1.5 ± 0.53 | 3.8 ± 1.1 |
| 10* | 1.1 ± 0.18 | 0.69 ± 0.23 | 1.6 ± 0.34 | 1.4 ± 0.35 | 2.2 ± 0.64 |
| 14 | 0.40 ± 0.14 | 0.32 ± 0.08 | 1.9 ± 1.02 | 0.43 ± 0.10 | 3.3 ± 0.86 |
| 21* | 1.02 ± 0.49 | 0.98 ± 0.30 | 2.9 ± 0.45 | 0.88 ± 0.19 | 5.0 ± 1.0 |
| Combined† | 1.2 ± 0.22 | 1.0 ± 0.20 | 2.3 ± 0.31 | 1.2 ± 0.18 | 3.4 ± 0.46 |

Values expressed as SI ± SEM. Controls not included in analysis.

* $p < 0.05$.

† $p < 0.001$ by MANOVA.

df = 3/12 at each test day.

take did not have skin on gross examination. In addition, repeat sections of 22 negative histologic specimens revealed epidermal elements in five, for a 23% false-negative rate.

MLR. The overall results of unidirectional MLR are shown in Table II. Allo FT resulted in a significantly increased SI ($p < 0.05$) at postgraft days 5, 7, 10, and 21 when compared with experimental groups. However, Allo CK was not significantly greater than Sham or Auto CK at any postgraft day. The time course of MLR response is shown in Fig. 1. There was an apparent overall decrease in the SI at day 10; then the SI for Allo FT showed a steady increase and reached a maximum at day 21. Comparison of the combined MLR data is shown in Fig. 2. Allo CK failed to induce the significantly elevated SI seen in Allo FT ($F[3/78] = 13.08$; $p < 0.001$). The SI for Allo CK was not significantly different than that for Auto CK or Sham. The mean SI for controls in each assay showed significant day to day variation and were consistently greater than for experimental groups, a finding reported by others using this model.⁸

Alloantibody formation. Serial dilutions of cytotoxic antibody titers for known positive control Allo FT is shown in Fig. 3. By postgraft day 10, Allo FT generated high alloantibody titers, with 50% cell killing (NIH score 4) seen at a 1/80 dilution. Cytotoxic antibody titers for each group as a function of postoperative

day is shown in Fig. 4. No cytotoxic antibody developed in any group during the first 5 days of the experiment. Alloantibody was first detected in Allo FT on postgraft day 7. Antibody titers increased steadily, reached positive scores (≥ 4) by postgraft day 10, and persisted for the duration of the experiment. By contrast, Allo CK did not generate cytotoxic antibody titers significantly greater than Auto CK or Sham.

DISCUSSION

In this study we demonstrated that CK allografts fail to induce sensitization to MHC alloantigens, as measured by unidirectional MLR and complement mediated microcytotoxicity test. Sensitization to FT allografts is detected at 5 days after grafting and persists for the duration of the experiment.

The biologic and mechanical properties of CK autografts are being actively investigated,⁹ but the immunogenicity of CK allografts remains poorly characterized. Initial studies of CK allografts performed in human beings suggested that long-term graft take was possible.^{4,10} However, in these studies CK allografts were placed on wounds with viable recipient dermis, and no objective proof of donor CK survival was provided. Others reported either that CK allografts took initially but were eventually replaced by donor epidermis¹¹ or that donor keratinocytes could not be detected in healed wounds.¹² Some reported that CK allografts

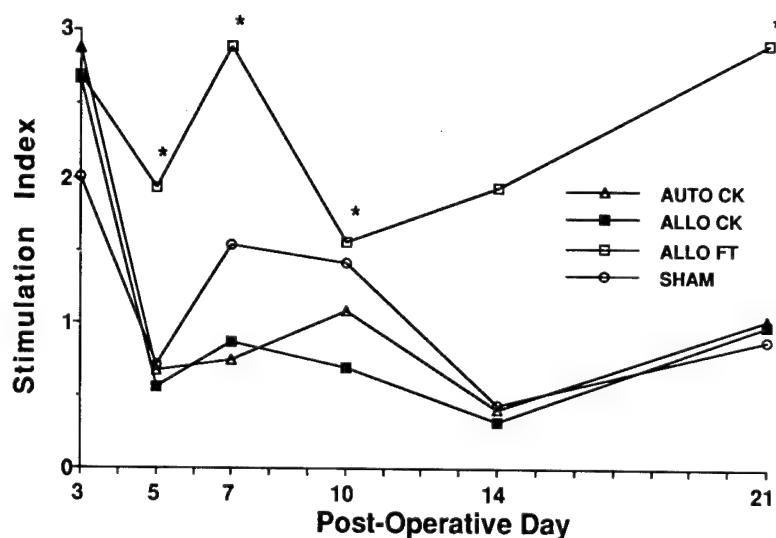


Fig. 1. Time course of MLR results. Each point represents the mean stimulation index for each group at each postgraft day. * $p < 0.05$ by MANOVA.

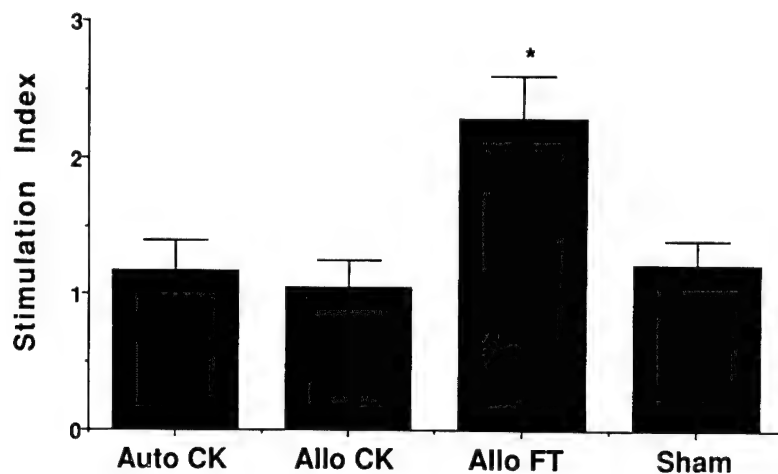


Fig. 2. MLR results for each group combined during all experimental days. * $p < 0.001$ by MANOVA.

were rejected outright, similar to split-thickness skin allografts.¹³ Similar conflicting observations have been reported in animal studies. Indefinite CK allograft take in mice, up to 70 days, was reported but no definite proof of donor CK survival was provided.¹⁴ Others reported that CK allografts were rapidly rejected in rats¹⁵ and in pigs.¹⁶

Determining the immunogenicity of CK allografts in vivo is difficult. Examination of grafts in situ is not always reliable, because many factors determine graft take. Successful CK grafting requires meticulous wound care because grafts are at substantial risk for trauma, infection, and desiccation. In addition, CK grafts are fragile. They consist solely of stratified layers of epithelial cells and lack the structural dermal elements present in normal skin. In several studies of human CK autografts under strict wound care guidelines, no more

than 50% take was seen in most patients. Thus graft take may not be a reliable indicator of graft immunogenicity. In addition, CK graft take is not easily assessed. Granulation tissue appears grossly similar to CK grafts, and biopsy findings that reveal epidermal cells do not distinguish donor keratinocytes from recipient epidermal cells that are present as a result of reepithelization of dermal wounds or wound contraction.

Dermal Langerhans cells, which are thought to be the predominant source of MHC class II antigens in native skin, do not persist in CK grafts; thus keratinocytes are the only cell type responsible for graft immunogenicity. Keratinocytes do not normally express MHC class II antigens.¹⁷ It has been proposed that CK allografts avoid stimulation of the immune system and subsequent rejection because of the lack of expression of foreign MHC class II antigens.¹⁰ But interferon- γ , a ubiqui-

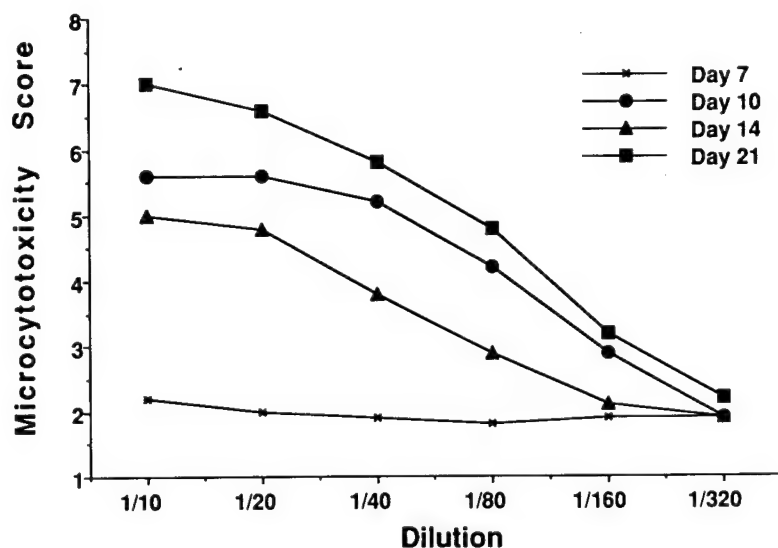


Fig. 3. Serial dilutions of positive control serum, Allo FT, as a function of NIH score (see text). A score ≥ 4 is positive. Each line represents the level of cytotoxic antibody generated by Allo FT for each day, from 7 to 21 days after grafting. By postgraft day 10, significant cytotoxicity is seen at dilutions up to 1/80.

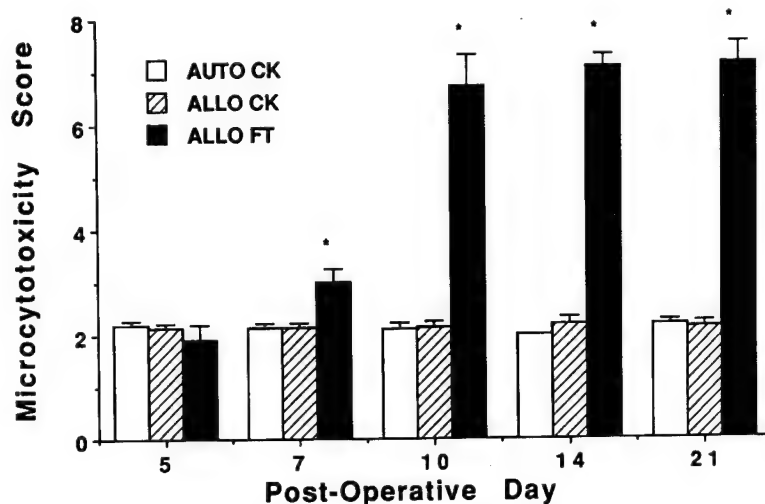


Fig. 4. Cytotoxic antibody levels in undiluted serum for each group as a function of postoperative day. NIH score ≥ 4 is positive and represents 50% cell killing. * $p < 0.05$ by MANOVA.

tous cytokine produced in response to injury and infection, is known to induce MHC class II antigen expression in keratinocytes within 3 days of exposure, both in vitro¹⁸ and in vivo,¹⁹ which may lead to class II alloantigen sensitization and eventual rejection.

MLR is a sensitive assay of potential allograft rejection that measures the degree of MHC class II incompatibility between two distinct lymphocyte populations.⁶ Both in vitro and in vivo sensitization are detectable by MLR. For instance, in secondary MLR assays, responders previously exposed to alloantigen exhibit an enhanced proliferative response after a second challenge to that antigen.⁶ In animals, prior expo-

sure to alloantigens results in an enhanced proliferative response,²⁰ and an increased MLR is observed in human beings during allogeneic skin graft rejection.²¹ Thus assessing MLR at fixed points after skin grafting effectively determines when or if sensitization to donor MHC class II antigens occurs. It is unknown how long CK allografts would need to persist to induce alloantigen sensitization. Allogeneic split-thickness skin grafts induce sensitization after only 48 hours of continuous contact with the recipient wound,²² presumably because of exposure to MHC class II antigens expressed on dermal Langerhans cells. Because keratinocytes rapidly respond to cytokines such as interferon- γ , they can po-

tentially express MHC class II antigens within 72 hours of grafting. Thus we expect that even with limited long-term CK graft take, sensitization by CK allografts should occur in this model.

Our MLR results are consistent with the findings of previous investigators who found that mouse¹⁴ and human²³ CK fail to stimulate allogeneic lymphocytes in vitro, as measured by the mixed skin lymphocyte response (MSLR) assay. In the MSLR assay CK grafts are disaggregated with trypsin and cocultured with allogeneic lymphocytes, and the proliferative lymphocyte response to alloantigen is measured. The MSLR is comparable to the traditional MLR and reportedly represents a similar immunologic reaction.²⁴ However, the MSLR does not measure in vivo sensitization by CK.

The complement mediated microcytotoxicity test is used to identify MHC class I antigens before organ transplantation and can also detect donor-specific alloantibodies after transplantation. We found that, unlike positive control FT allografts, CK allografts did not induce alloantibody formation. These results are consistent with previous findings that CK allografts did not induce alloantibody formation in human beings²⁵ or rats.¹⁵

Although we did not detect sensitization to donor MHC class I or class II antigens in animals after CK allografting, it is possible that mice were sensitized to other antigens that might contribute to allograft rejection. Improved methods of in situ graft examination, including the use of donor-specific DNA sequences or monoclonal antibodies to donor-specific cell surface antigens, will help define the immune response to CK allografts. In addition, other assays of immunogenicity, such as second set rejection, may be more accurate assessments of in vivo sensitization to CK allografts. Fully defining the immunogenicity of CK allografts might identify other antigens and mechanisms responsible for allograft rejection. Because CK grafts consist of a single cell type, genetic manipulation of the expression of specific surface antigens at the molecular level may be possible, thus creating an immunologically inert, functional graft.

In conclusion, CK allografts do not induce sensitization to MHC alloantigens in a murine model within 21 days, as measured by MLR and cytotoxic antibody formation. A continued careful and systematic effort to define the immunogenicity of CK is required if the clinical use of CK allografts for permanent wound coverage is to be realized.

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DISCUSSION

Dr. Charles B. Cuono (New Haven, Conn.). This was a very carefully executed study that provides us with some *in vivo* evidence that complements the *in vitro* work that Dr. Hefton did a few years ago.

A couple of questions arise regarding the use of allogeneic human keratinocytes as permanent epidermal replacement. The first of these relates to the issue to which you alluded in looking at the wounds, and that is the persistence of the keratinocytes themselves. Despite the initial flurry of interest sparked by Hefton and Thivolet, a number of investigators, including Irene Leigh, Tanya Phillips, and our own laboratory have clearly demonstrated, with specific probes, that these cultured allogeneic keratinocyte grafts do not persist. Is it possible that you did not see the MLR stimulation because you did not have a sufficient dose of alloantigen present in this particular model? Or, to look at it another way, how can you confirm that keratinocyte allografts persist over time?

The second question, to which your model is very well suited, is what has been called the "time bomb" possibility with keratinocyte allografts; namely, although they do not constitutively express MHC class II antigens, they can easily be induced to express them through interferon- γ stimulation. An intercurrent viral infection in a patient receiving these grafts or a walk in the woods and getting poison ivy can induce interferon- γ and induce class II expression of keratinocyte allografts and trigger rejection. It would be very important to answer this question, and your model is uniquely suited to do that.

Dr. Raymond Pollak (Chicago, Ill.). Much like corneal epithelium, the use of cultured tissue is deficient in class II antigen-presenting cells. If you subscribe to the theory of Larson and Austyn that class II antigen-presenting cells are crucial to triggering rejection responses, this is a very elegant demonstration that, in fact, in the absence of class II antigen-presenting cells, you do not get an immune response. Did you stain your cultured epithelium to look for these class II cells specifically, because rat tail skin is deficient in class II antigen-presenting cells? In your full-thickness graft as controls,

did that come from the tail skin or did it come from elsewhere on the animal?

Dr. David B. Herndon (Galveston, Texas). Because your study was only 21 days, how can you be sure that sufficient interface has been developed in that time for antigenicity to be recognized systemically, particularly because no dermal elements have proliferated at that time, even if the substance is persistent?

Dr. Cairns (closing). Dr. Cuono's observation that culture keratinocyte allografts have not been shown to persist in human beings is representative of one of the major problems we face when studying this material. Several investigators have reported that cultured keratinocyte autografts do not persist in more than 50% of patients, and in many of the earlier studies that investigated graft immunogenicity *in vivo*, cultured keratinocyte allografts were placed on wounds with viable, recipient dermis. One of the potential functions of keratinocyte grafts, if biologically active, would be to secrete cytokines and stimulate wound healing, resulting in recipient dermis regenerating epidermal elements, and it would not be surprising that this regenerated epidermis would eventually replace allogeneic grafts. The reason we used this model is because we placed cultured keratinocyte allografts on wounds that clearly did not have underlying recipient dermis and thus limited the ability of wounds to displace grafts with regenerated epidermal elements.

Concerning questions about the potential dose and length of exposure to alloantigens, Ballantyne showed several years ago in another skin graft model that sensitization to alloantigens occurs by imbibition within 48 hours after graft placement. Although it is not assured that we induced sensitization to alloantigens in our model, we proposed that with evidence of graft take, the potential for sensitization was substantial and that if it did occur, we would be able to detect it.

An interesting feature of cultured keratinocyte allografts is that they do not normally express class II antigens but can be induced to do so with interferon- γ . We are currently examining *in vivo* class II antigen expression in these grafts. Because grafts are depleted of class II antigen-presenting cells, which we did not stain for in this study but have been demonstrated previously by others, it is possible that any class II antigens expressed in these grafts lead not to a rejection response but to something more closely akin to clonal anergy. To answer this and other questions, we hope to improve models that study class II antigen expression and improve models that study their resultant effects, including, for example, assessment of second set rejection.

In these studies we grafted positive controls with allogeneic full-thickness flank skin grafts. Although tail skin consists of about 0.3% Langerhans' cells, which is a little less than that observed in flank skin, keratinocyte expression of class II antigens in both types is relatively comparable.

CULTURED MOUSE KERATINOCYTE ALLOGRAFTS PRIME FOR ACCELERATED SECOND SET REJECTION AND ENHANCED CYTOTOXIC LYMPHOCYTE RESPONSE^{1,2}

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It has been reported that cultured keratinocyte (CK) allografts are not rejected in mice, unlike in other species. Several reports have suggested that mouse CK allografts are incapable of stimulating a primary alloresponse, including sensitization of recipients to alloantigens. In this study, we investigated the immunogenicity of mouse CK allografts *in vivo* by determining whether CK allografts primed for a second set rejection response. First, we grafted mice with either CK allografts, CK autografts, full-thickness (FT) allografts, or no graft at all. We then regrafted mice 4 weeks later with a tail skin allograft. Mice grafted with CK allografts rejected second allografts as rapidly and as vigorously as mice grafted with FT flank allografts. Next, we tested whether CK allograft primed recipients for enhanced CTL responses. We found that mice grafted with CK allografts generated a significantly enhanced CTL alloreactive response after *in vitro* stimulation. The response was similar to that of mice grafted with FT skin allografts. With evidence that CK allografts primed, we biopsied wounds immediately after CK allografting and, using Western immunoblotting, found that CK allografts had substantial expression of MHC class II antigens *in vivo*. We conclude

from the results of our studies that mouse CK allografts unequivocally prime recipients to alloantigens *in vivo* and suggest that a possible mechanism for alloantigen priming may be CK allograft expression of MHC class II antigens.

The immunogenicity of cultured keratinocyte (CK)* allografts has been a subject of controversy for nearly a decade. In the mouse, unlike other species, such as rat (1), pig (2), rabbit (3), and human (4), it has been reported that CK allografts survive indefinitely (>70 days) (5-7). The theoretical basis provided for this finding is the "passenger leukocyte" hypothesis of allograft rejection, which suggests that since Langerhans cells (LC), the predominant APC and source of MHC class II antigens in skin, do not persist in CK cultures (5), mouse CK allografts should fail to stimulate recipient T cells and initiate a subsequent allograft rejection response (8). It should be noted, however, that replacement of donor LC with recipient LC does not ameliorate rejection (9). In addition, this theory does not explain why LC-free CK allografts in other species are rejected, or at least explain why they fail to survive permanently. An excellent overview concerning this controversy has been published previously (10).

A possible explanation for this seeming discrepancy is that unlike CK in other species, mouse CK grafts reportedly do not express MHC class II antigens *in vitro*, even under the influence of IFN- γ (11). Further studies suggest that this lack of MHC class II expression results in the inability of CK allografts to stimulate allogeneic lymphocytes *in vitro* (12), allows CK allografts to survive in animals previously sensi-

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² The viewpoints expressed herein are those of the authors and do not necessarily represent those of the U.S. Army or Department of Defense.

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* Abbreviations: ALLO, allograft; AUTO, autograft; CK, cultured keratinocyte; FT, full thickness; LC, Langerhans cell; MLR, mixed lymphocyte reaction; MST, median survival time.

tized by full-thickness (FT) allografts (7), and even allows for the induction of tolerance to FT allografts (13).

A recent study (14) reported that CK allografts appear to survive indefinitely in naive mice, but are rejected in mice presensitized with FT skin grafts. They also showed that although CK allografts did not induce tolerance as determined by mixed lymphocyte reaction (MLR), they were lysed by alloreactive CTL. Previously, we demonstrated that mouse CK allografts do not induce cytotoxic alloantibody or enhanced MLR responses (15). However, our studies did not rule out the possibility that CK allografts primed and did not examine the possibility that they induced tolerance.

Resolving the issue of sensitization is important not only because of its implications on our understanding of the response to alloantigens, but because several investigators are advocating the use of CK allografts in humans as temporary dressings that accelerate wound healing (16–21). If CK allografts sensitize, however, there is a potential that after multiple applications these grafts could elicit a second set rejection response resulting in a state of chronic rejection and inflammation in the wound that could ultimately delay wound healing, thereby eliminating any potential clinical benefit associated with their use.

In the present study, we investigated whether mouse CK allografts prime (or induce tolerance) for accelerated second set rejection of FT allografts and enhanced CTL alloreactivity. In addition, we examined a potential mechanism for sensitization by using Western immunoblotting to analyze MHC class II antigen expression by CK allografts *in vivo*.

MATERIALS AND METHODS

Mice. Eight- to 10-week-old CBA/J (CBA, H-2^k) (Harlan Sprague Dawley, Inc., Indianapolis, IN) and C57BL/6 (B6, H-2^b) (Charles Rivers Laboratories, Wilmington, MA) female mice were used. Mice were maintained in our animal facilities and all animal protocols were approved by the University of North Carolina Committee on Animal Research, in accordance with the NIH. CBA mice used as primary graft recipients were randomized into 4 groups and received flank grafts of either CBA CK (autograft [AUTO] CK), B6 CK (allograft [ALLO] CK), B6 FT (ALLO FT), and no graft (CBA).

CK culture. Graft donor mice were killed by methoxyflurane euthanasia (Metofane; Pittman-Moore, Chicago, IL). Tail skins were washed with 70% ethanol, harvested, and stored overnight in Dulbecco's modified MEM (DMEM; Lineberger Cancer Center, Chapel Hill, NC) supplemented with 0.1% penicillin/streptomycin (Lineberger Cancer Center). Skin was then finely minced and incubated in 0.25% trypsin (Sigma Chemical Co., St. Louis, MO) solution for 1.5 hr at 37°C. Epidermal cells were separated from dermis by mechanical agitation and 4×10^6 cells were cocultured at 5% CO₂, 37°C with 2×10^6 growth-inhibited (4 µg/ml mitomycin-C, Sigma) mouse connective tissue L cells (LTK⁻, H-2^k, ATCC CCL 1.3), used as feeder layers, in a solution of DMEM and Ham's F-12 (Lineberger Cancer Center), supplemented with 5% FBS (HyClone Laboratories Inc., Logan, UT), hydrocortisone 0.4 µg/ml, transferrin 5.0 µg/ml, and insulin 5.0 µg/ml (all from Sigma), cholera enterotoxin 0.01 µg/ml (Schwartz Mann/ICN Biochemical Inc., Costa Mesa, CA), and amphotericin 5.0 µg/ml (E.R. Squibb & Sons Inc., Princeton, NJ). Epidermal growth factor, 10.0 ng/ml (Collaborative Research Inc., Bedford, MA), was added with the first media change. The media were changed as needed (average: 3 changes/week). One week after plating, LTK⁻ cells were removed from culture with differential trypsinization (0.1% trypsin for 5 min) and keratinocytes were grown to confluence (about 14–17 days).

Primary flank skin grafting. After reaching confluence, CK grafts were dispased (Dispase, 1.2 U/ml; Boehringer Mannheim, Indi-

anapolis, IN) and placed on petrolatum gauze, basal side up, and stored at 37°C until ready for grafting. Recipient mice were anesthetized with methoxyflurane. The torso was circumferentially shaved and a 3 cm² area of skin on the left flank was excised, leaving the underlying panniculus carnosus intact. The gauze-backed CK graft was placed basal side down onto the flank wound and tucked under the surrounding wound edge. The wound was covered with Vigilon (C.R. Bond, Inc., Berkeley Heights, NJ) and a stretch fabric bandage secured with skin staples. Bandages were removed 7–10 days after grafting. FT skin grafts were obtained from the dorsum of separate donors and grafting was performed in a procedure similar to that of CK grafts.

Secondary FT skin tail grafting. Four-to-five weeks after flank grafting, recipient mice were anesthetized with methoxyflurane. The tail was washed with ethanol and two 5-mm FT skin grafts (1 CBA autograft and 1 B6 allograft) were placed on the tail dorsum, using a previously described method (22). Tail grafts were protected with a specially manufactured glass tube to prevent trauma and to keep grafts free of debris. Tail tubes were removed 2–3 days after grafting and only technically successful grafts (grafts that were present, vascularized, viable, and not grossly infected at the time of tube removal) were assessed for viability daily. Viability was determined by scoring grafts for redness, dryness, scaliness, and the presence and quality of hair, with rejection arbitrarily defined by 2 independent observers. The arithmetic median graft survival time (MST) for each group was then determined. Results were compared using Wilcoxon rank and chi-square analysis.

CTL alloreactivity. To assess whether CK allografts induce enhanced alloreactivity, graft recipient splenocytes were isolated 14–17 days after grafting and used as effectors in primary CTL assays. Additional recipient splenocytes (2×10^6 cells/cc) were cocultured with irradiated (2000 rads) B6 donor splenocytes (2×10^6 cells/cc) in RPMI 1640 media supplemented with 10% FBS, 5×10^{-5} M 2-ME (Sigma), and 0.1% penicillin/streptomycin. After 3, 4, 5, and 6 days of coculture with allogeneic splenocytes, effector lymphocytes were harvested, washed in media, and assayed for CTL activity. Target cells for CTLs were LTK⁻ (H-2^k) and EL-4 mouse lymphoma cells (EL-4, H-2^b, ATCC TIB 39). Targets were incubated with 100 µCi of Na₂⁵¹CrO₄ (ICN Biomedicals Inc., Irvine, CA) for 30 min at 37°C, then washed 3 times and counted. Targets were added to wells of a round-bottom 96-well plate at 1×10^4 cells/well and cocultured, with increasing number of CTL effectors (1.25×10^5 – 1.5×10^6 cells/well) in a total volume of 150 µl of RPMI 1640 medium containing 10% FBS. After 4-hr incubation at 37°C, supernatant from each well was harvested and the radioactivity was counted. Percentage of cell lysis was calculated according to the formula: % specific lysis = $[(\text{cpm}_{\text{sample}} - \text{cpm}_{\text{spontaneous}}) / (\text{cpm}_{\text{maximum}} - \text{cpm}_{\text{spontaneous}})] \times 100$. Spontaneous release represents the radioactivity released by target cells in the absence of effectors. Maximum release represents the radioactivity released by targets lysed with 5% Triton X-100. The average spontaneous release in target cells as a percentage of the maximum release ranged from 6% to 9%. All assays were tested in duplicate and means were shown.

H-2^b MHC class II antigen determination. H-2^b MHC class II antigen expression in CK allografts was assayed by Western immunoblotting analysis using KL295, an mAb that reacts with denatured β chains of H-2^b MHC class II antigens, but not H-2^k MHC class II antigens (23). On day 2–7 after CK allografting, a 3-mm punch biopsy of grafted wounds was obtained and lysates were prepared by solubilizing biopsies in 200 µl of 0.01 M Tris-HCl (pH 7.4), 0.150 M NaCl, 0.5% NP-40, and 1 mM PMSF (23). After vortexing, pellets were incubated on ice for 15 min, then centrifuged at 4°C for 15 min and stored at –20°C. Western immunoblots were performed as described previously (23); twelve percent SDS polyacrylamide gels were poured using a Bio-Rad model 360 mini-vertical slab-cell and model 361 casting chamber. After normalizing for total protein concentration, detergent-extracted biopsies and molecular markers were separated on one-dimensional gels and electroblotted onto nitrocellulose paper. Blots were blocked with 1% BSA in Tris-HCl (pH

8.0). After incubation with the primary mAb, specific proteins were detected using goat anti-mouse alkaline phosphatase-labeled IgG. Relative expression of MHC class II antigen expression was performed using video densitometry.

RESULTS

Second set rejection. To determine whether CK allografts prime recipients for second set rejection, we grafted mice with CK grafts, then 4 weeks later challenged them with FT skin grafts. To ensure that results from the second set rejection experiments were reliable, we controlled for potential factors that might influence the immunologic response to alloantigens. Since we observed previously that fibroblast feeder layers can persist in CK cultures (unpublished data), we eliminated the possibility of feeder layer allosensitization by using H-2^k haplotype (LTK cells) feeder layers for all CK grafts grafted onto CBA (H-2^k) mice. No attempt (i.e., biopsy) was made to determine the fate of the flank graft and wounds were disturbed as little as possible. By the time mice received the FT tail graft, recipient wounds in all groups had contracted and had closed completely. Finally, to ensure that second set graft loss was a result of rejection and not due to technical or graft failures, all mice received allogeneic and autologous tail grafts and only those with technically successful autografts at postgraft day 3 were included in the analysis. The results show that both ALLO FT and ALLO CK prime for accelerated rejection, as shown in Table 1. The MST of the FT tail allograft for the ALLO CK and ALLO FT groups was 9 days ($X^2=1.0$, $P=0.3$). However, the MST for both the AUTO CK and CBA groups was 13 days ($X^2=1.6$, $P=0.2$), and the MST for both ALLO CK and ALLO FT was significantly less than that for AUTO CK and CBA ($X^2>18$, $P<0.01$). Though it appears that ALLO CK rejected the tail allograft at a slightly slower rate than ALLO FT, the difference was not significant. We conclude from this experiment that CBA mice were primed for accelerated rejection of FT allografts by B6 CK allografts in vivo. A repeat experiment yielded identical results.

CTL reactivity. To determine whether we could detect a change in the kinetics of induction of CTL, we examined the induction of alloreactive CTL in vitro after in vivo priming. CBA recipient splenocytes were isolated 2–3 weeks after flank grafting and were used as effectors in primary CTL assays performed at the time of isolation (day 0) or after 3–6 days of in vitro culture with irradiated B6 splenocytes. Targets were either MHC-matched LTK⁻ (H-2^k) or allogeneic EL-4 (H-2^b). On day 0, as expected, the CTL response for all groups, including ALLO FT, was undetectable (data not shown). By day 3, however, CTL alloreactivity was detected in ALLO FT (Fig. 1). By day 4, CTL alloreactivity in both ALLO FT and ALLO CK was substantially greater (50–60% vs. 32% at E:T ratio of 150) than that of AUTO CK and CBA

(Fig. 2) and remained greater until the CTL alloreactivity in all groups was greater than 50% at day 6 (Fig. 3). These results indicate that as expected from the skin graft data, both ALLO FT and ALLO CK recipients re-exposed to alloantigen in vitro develop enhanced alloreactivity, providing further evidence that ALLO CK primes graft recipients for a second set rejection response.

MHC class II antigen detection. With evidence that ALLO CK primes, we investigated whether in vivo MHC class II antigen expression by mouse CK may be responsible for alloantigen sensitization by providing MHC class II as well as MHC class I molecules. We biopsied ALLO CK graft recipients 2, 3, 5, and 7 days after grafting and assessed them for H-2^b MHC class II antigen expression using Western immunoblotting (Fig. 4). There was no detectable baseline expression of H-2^b MHC class II antigen in ALLO CK in vitro. However, there was substantial H-2^b MHC class II antigen expression by ALLO CK in vivo. By video densitometry, it peaked 3 days after grafting and persisted up to at least 7 days after grafting (Table 2). This experiment was repeated with identical results.

DISCUSSION

In this study, we demonstrate that mouse CK allografts sensitize graft recipients to alloantigens as detected by accelerated rejection of second, FT tail allografts and by enhanced CTL alloreactivity. Furthermore, we provide Western immunoblot evidence that CK allografts express MHC class II antigens in vivo.

Determining the immunogenicity of CK allografts has been a vexing problem, both in animal models and in human studies. In the mouse, Worst et al. (24) reported prolonged survival of CK allografts and attributed this long-term survival to the lack of Ia antigens on epidermal cells. Several studies subsequently reported long-term survival of mouse CK allografts (5–7, 25). These authors stressed that special care had to be taken to avoid recipient wound re-epithelization because of the rodents' remarkable wound contractile ability. Therefore, silicone chambers were devised to restrict wound contraction and CK grafts were placed in these chambers and the status of the transplanted graft was analyzed. Despite histologic evidence of keratinocytes in the wounds, little or no definitive evidence of donor-specific CK graft survival was provided in these studies. In our previous report, we found that it was very difficult to prove definitively that donor CK graft (either autologous or allogeneic) survived in mice, as neither histologic nor gross examination were reliable indicators of graft survival (15). The use of alloantigen-specific immunostains could theoretically provide definitive evidence of donor keratinocyte survival. We have found, however, that the reliability of this technique in detecting donor-specific keratinocyte antigens in our grafting system is limited.

An indirect, but perhaps more reliable, method of determining immunogenicity is to examine the antigenicity of CK in vitro or in vivo. Investigators have reported that mouse CK fail to stimulate naive lymphocytes in the mixed epidermal lymphocyte reaction (5, 14), and attributed this response to a lack of MHC class II antigens on CK allografts. In a modification of this concept, we studied whether mice were primed after CK allografting by performing MLR 3–21 days after grafting. We found that the MLR was no greater than control (15). However, the results from these studies are inconclu-

TABLE 1. Second set graft survival

| Flank graft | Tail allograft survival ^a (days) | MST (days) |
|----------------|--|---------------|
| ALLO FT (n=21) | 7, 8(6), 9(7), 10(6), 11 | 9 |
| ALLO CK (n=22) | 8(2), 9(9), 10(8), 11, 13(2) | 9 |
| AUTO CK (n=17) | 11(2), 12(3), 13(7), 14(4), 16 | 13 |
| CBA (n=11) | 9, 11, 13(6), 14(3) | 13 |

^a Number of days graft survived is bold; number in parentheses is number of grafts rejected at that day.

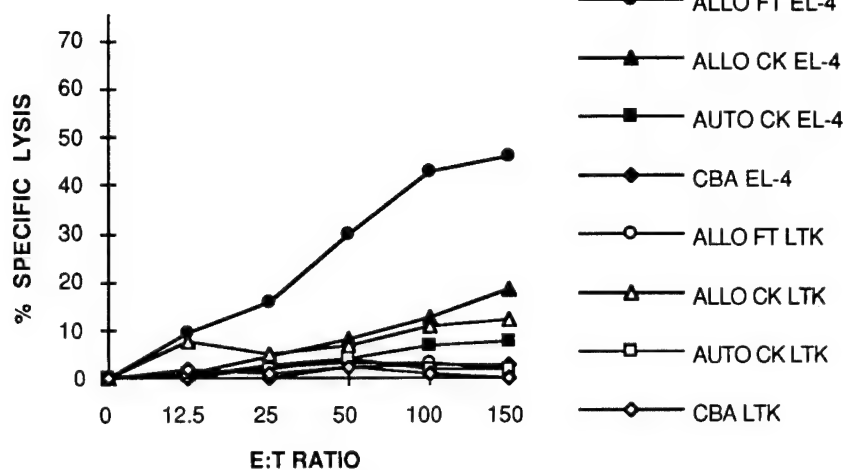


FIGURE 1. Recipient CTL after 3 days of in vitro allostimulation.

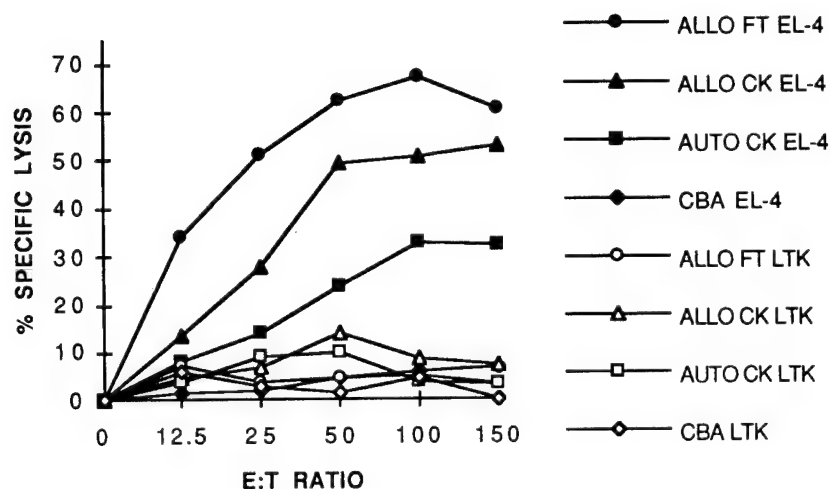


FIGURE 2. Recipient CTL after 4 days of in vitro allostimulation.

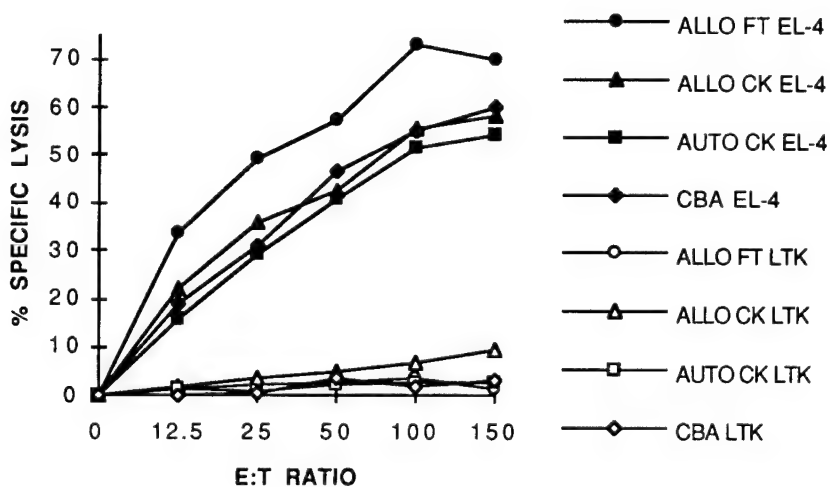


FIGURE 3. CTL alloreactivity after 6 days of in vitro allostimulation.

sive, because these assays may not be sensitive enough to detect sensitization in vivo. The best method to detect allosensitization is to determine whether CK allografts induce a second set rejection response, as originally described by Medawar (26). Others have performed priming experiments similar to the one we describe in this study (with important differences), yet obtained different results. In one study, ir-

radiated CK allografts were injected intraperitoneally and no effect on second FT allograft survival was found (5). Another study (7) reported that CK allografts also did not induce sensitization, but this study also found that CK allografts were not rejected, even in the presence of ongoing allogeneic rejection, a result that has not been confirmed by others (14). Finally, one group reported that CK allografts induced a pro-

BURN INJURY SELECTIVELY IMPAIRS HOST SENSITIZATION TO CULTURED KERATINOCYTE ALLOGRAFTS

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Suzan deSerres, BA, Lisa A. Brady, BS, and
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CULTURED KERATINOCYTE (CK) allografts have been proposed as a method of early wound coverage for the massively burned patient.¹ The decreased immunogenicity of these grafts may limit rejection and is explained by the findings that CK allografts lack passenger leukocytes, do

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not constitutively express major histocompatibility class II antigen (MHC II Ag), and fail to generate allospecific antibody once grafted.² We have recently shown, however, that CK allografts express MHC II Ag in vivo and prime nonburned hosts for accelerated second-set rejection via enhanced cytotoxic T-cell response.³ Such sensitization may account for the observation that CK allografts have limited long-term survival, with gradual replacement by host keratinocytes in partial-thickness wounds.⁴ However, burn injury produces significant immune dysfunction, which may affect alloantigen sensitization. In this study, we investigated the effect of burn injury on CK allograft sensitization to determine if the immunosuppression of burn injury may lead to graft tolerance.

MATERIALS AND METHODS

Ninety-nine female CBA mice received either a sham or 20% full-thickness (FT) contact burn. Flank wounds were partially excised 72 hours later and were grafted with FT C57BL/6 (B6) allograft (Allo FT), CK B6 allograft (Allo CK), or CK autograft (Auto CK). CK grafts had previously been grown to confluence after coculture with a growth-arrested, autologous fibroblast feeder layer that had been removed through trypsinization. Two weeks after flank grafting, mice were challenged with FT tail allografts. Technically successful second-set grafts were observed for rejection by assessing changes in color, scale texture, and hair orientation. Median graft survival time (MST) was compared between groups by Wilcoxon rank and chi-square analyses.

RESULTS

Survival curves for second-set tail grafts are represented in Figure 1, and the MST for each group can be found in the accompanying legend. As noted previously, both CK and FT allografts sensitized normal mice with equal efficacy. Second-set rejection in both groups occurred at 9 days ($P = 0.73$), compared to the Auto CK group, which had rejection at 13 days ($P < 0.0001$). Burn injury significantly delayed second-set rejection in all three grafting groups, increasing tail graft survival in BURN Auto CK by 1 day ($P < 0.01$), in BURN Allo FT by 1 day ($P < 0.05$), and in BURN Allo CK by 2.5 days ($P < 0.001$). BURN Allo CK still had accelerated tail graft rejection when compared to BURN Auto CK (11.5 vs. 14 days, $P < 0.001$), but BURN Allo CK showed significantly delayed priming when compared to BURN Allo FT (11.5 vs. 10 days, $P < 0.01$).

CONCLUSIONS

Burn injury, with its associated immune dysfunction, prolongs both primary and second-set allograft survival. Although both CK and FT allo-

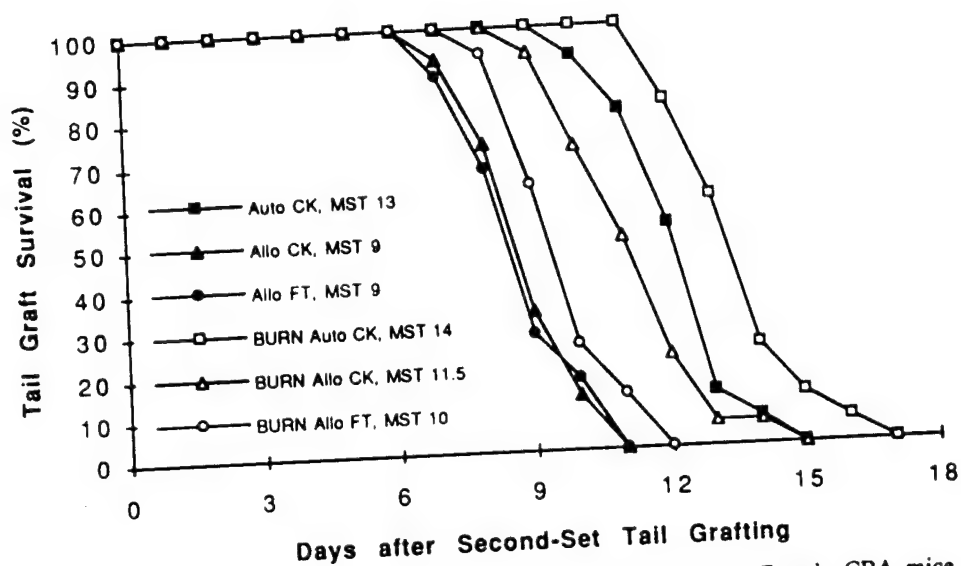


Fig 1—The effect of burn injury on second-set tail graft survival. Female CBA mice received either a sham or 20% BURN injury, followed 3 days later by partial wound excision and flank grafting with autologous cultured keratinocytes (Auto CK, $n = 32$), allogeneic cultured keratinocytes (Allo CK, $n = 33$), or allogeneic full-thickness skin (Allo FT, $n = 34$). Two weeks after grafting, all animals were challenged with an allogeneic full-thickness tail graft. Median tail graft survival time (MST) for each group can be found in the legend.

grafts prime the unburned murine host with equal efficacy, burn injury causes greater impairment of CK allograft sensitization. Understanding and manipulating the mechanism of impaired sensitization may improve the long-term survival of CK allografts and thereby permit their use in early, permanent burn wound coverage.

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**XENOGENEIC MOUSE FIBROBLASTS PERSIST IN
HUMAN CULTURED EPIDERMAL GRAFTS:
A POSSIBLE MECHANISM OF GRAFT LOSS**

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Cultured epidermal autografts (CEA) have been used for a variety of conditions as a permanent wound cover since they were first introduced as a method of burn wound closure nearly 15 years ago.^{1, 2} While the success of CEA has been variable, coverage of the massive burn wound continues to be the most common indication for the large scale use of CEA.^{3, 4} Unfortunately, several centers have recently reported disappointing results with CEA, particularly in the massively burned patient.⁵⁻⁷ Many factors have been recognized as potential causes of CEA loss, including poor status of the pre-grafted wound, inherent graft fragility, and graft bed infection.²⁻⁴ Several investigators, however, have reported characteristics of CEA loss that suggest that an immunologic reaction, similar to allograft rejection, may also cause graft loss.^{7, 8} In these reports several patients developed an inflammatory process in wounds that had initially healed after grafting with CEA. This inflammatory response, which occurred in the absence of any demonstrable wound infection or obvious trauma, resulted in focal areas of graft blistering and destruction, and frequently resulted in total CEA loss.^{7, 8}

Since CEA are derived from autologous tissue, the only obvious sources of antigens that could initiate this type of immunologic reaction are the media and feeder layers used to develop CEA. Nearly all clinically available CEA, including commercially produced CEA, are developed using modifications of the methods originally described by Rheinwald and Green.^{4, 9-11} This system generally requires the use of fetal bovine serum (FBS) in the media and growth-arrested 3T3 Swiss mouse (3T3) fibroblast feeder layers to rapidly and efficiently generate CEA.¹¹ It is possible, despite extensive efforts to remove them, that either or both of these components could persist in CEA and generate the immunologic response observed in some patients.

Our laboratory has demonstrated previously that patients grafted with CEA generate increased levels of antibody to FBS, suggesting that this xenogeneic

antigen persists in CEA.¹² With recent developments in monoclonal antibody technology, it is now possible to determine whether 3T3 fibroblasts persist in CEA. In this study, we examine whether 3T3 fibroblasts persist in multiple passage CEA, and whether these fibroblasts can be stimulated to produce transplantation antigens, specifically major histocompatibility (MHC) class II antigens, in response to interferon-gamma (IFN- γ) stimulation.

METHODS:

Cultured Epidermal Grafts

Discarded, unused remnants of split-thickness skin grafts obtained from randomly selected patients admitted to the Jaycee Burn Center were used as initiators of the epidermal cultures. All specimens were obtained with approval of the Institutional Review Board of the School of Medicine at the University of North Carolina-Chapel Hill.

The method of epidermal culture has been described elsewhere, and is similar to that of Rheinwald and Green.¹²⁻¹⁴ Partial thickness skin was stored overnight in Dulbecco's modified MEM (DMEM, Lineberger Cancer Center, Chapel Hill, NC) supplemented with 0.1% penicillin/streptomycin (Lineberger Cancer Center, Chapel Hill, NC). Skin was then incubated in 0.25% trypsin (Sigma Chemical Co., St. Louis) solution for 1.5 hours at 37°C. Epidermal cells were separated from dermis by mechanical agitation and 2×10^6 cells were co-cultured at 5% CO₂, 37°C with 1×10^6 growth-inhibited (cells cultured at 5% CO₂, 37°C with 4 μ g/ml mitomycin-C (Sigma Chemical Co., St. Louis, MO) in HBSS (Lineberger Cancer Center) for 2 hours, then washed x3 with HBSS and pipetted into a single cell solution with 0.1% trypsin) NIH 3T3 Swiss albino mouse embryo fibroblasts (3T3, ATCC CRL 1658) or with mouse connective tissue L cells (LTK⁻, ATCC CCL 1.3) used as feeder layers, in a solution of DMEM and Ham's F-

12 (Lineberger Cancer Center), supplemented with 5% FBS (HyClone Laboratories Inc., Logan, UT), hydrocortisone 0.4 μ g/ml, transferrin 5.0 μ g/ml, and insulin 5.0 μ g/ml (all from Sigma Chemical Co., St. Louis, MO), cholera enterotoxin 0.01 μ g/ml (Schwartz Mann/ICN Biochemical Inc., Costa Mesa, CA), and amphotericin 5.0 μ g/ml (E.R. Squibb & Sons Inc., Princeton, NJ). Epidermal growth factor, 10.0ng/ml (Collaborative Research Inc., Bedford, MA), was added with the first media change. The media was changed as needed (average: three changes per week). One week after plating, 3T3 or LTK⁻ fibroblasts were removed from culture by differential trypsinization supplemented with EDTA (0.1% trypsin with 0.02% EDTA for 5 minutes) and epidermal cultures were grown to confluence (about 14-17 days). After reaching confluence, CEA were either processed as primary cultures in order to detect persistent fibroblasts by fluorescent activated cell sorting (FACS) and Western immunoblotting or passed into secondary cultures at a density of 2×10^6 cells/100mm². Media changes were performed as before and when secondary cultures reached confluence, they were either passed into tertiary cultures (2×10^6 cells/100mm²) or processed for fibroblast analysis.

Fluorescence activated cell sorting (FACS) analysis

CEA grown with 3T3 were disaggregated to a single cell suspension by trypsinization. Cells were placed in a 96 well plate at 2×10^5 cells per well and kept on ice for the duration of the experiment. Human keratinocytes were spiked with 3T3 cells at 10% and 2% of total cell number to serve as controls. Cultures were incubated for 30 minutes with monoclonal rat antibody specific for murine MHC Class I (Rat IgG2a, ATCC TIB 126, American Type Culture Collection, Rockville, MD). They were washed three times in PBS with 3% FBS and 0.05% NaAzide. They were subsequently incubated for 30 minutes with affinity-purified

biotinylated goat anti-rat IgG (H&L, 3050-08, Southern Biotechnology Associates, Inc., Birmingham, AL), and washed as before. Cells were stained for FACScan detection by a 30 minute incubation with streptavidin-FITC conjugate (7100-02, Southern Biotechnology Associates, Inc., Birmingham, AL), followed by a final series of three washes. Two negative controls were used, the first substituting PBS with 3% FBS for the primary antibody (no 1°), and the second substituting rat anti-Human HLA DR5 (ATCC HB 151, American Type Culture Collection, Rockville, MD) for the anti-Class I antibody (irrelevant rat monoclonal antibody). Immediately prior to FACS analysis, propidium iodide (PI) [0.5mg/ml in acetate-EDTA buffer (0.1M NaAcetate, 2g/l Na₂EDTA, pH 5.2)] was added to the sample at a concentration of 10µl PI per 500µl of sample, and gating was set to eliminate the majority of dead cells. 3T3 fibroblasts were used to set the positive analysis gate that encompasses the positive shift produced by staining murine cells with antibody to MHC class I. Cells were analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Cells were excited at 488nm with an air cooled argon laser, using a 530nm band-pass emission filter for detection of the FITC signal and a 590nm long-pass emission filter for detection of the PI. Positive staining cells were calculated by the following formula:
$$\frac{\text{HK}[(\text{cells positive with specific antibody}) - (\text{cells positive with irrelevant antibody})]}{3\text{T3}[(\text{cells positive with specific antibody}) - (\text{cells positive with irrelevant antibody})]}$$

Western Immunoblotting

Mouse fibroblast MHC class II antigen expression in CEA was assayed by Western immunoblotting analysis using a monoclonal antibody (KL295) that reacts with denatured β chains of 3T3 fibroblast MHC class II antigens, but not with LTK⁻ nor human MHC class II antigens.^{15, 16} To enhance potential mouse MHC class II antigen expression, cultures were treated with recombinant murine IFN- γ

(10,000 Units/ml; a generous gift of Genentech, Inc., San Francisco, CA) for four days prior to harvest. Following trypsinization, culture lysates were prepared by solubilization in 200 μ l of 0.01 M Tris-HCl, pH 7.4, 0.150M NaCl, 0.5% NP-40, 1mM PMSF.¹⁵ After vortexing, pellets were incubated on ice for 15 minutes, then centrifuged at 4°C for 15 minutes and stored at -20°C. Western immunoblots were performed as previously described;^{15, 17} 12% SDS polyacrylamide gels were poured using a Bio-Rad Model 360 mini vertical slab-cell and Model 361 casting chamber. After normalizing for total protein concentration, detergent extracted biopsies and molecular markers were separated on one-dimensional gels and electroblotted onto nitrocellulose paper. Blots were blocked with 1% BSA in Tris-HCl, pH 8.0. Following incubation with the primary monoclonal antibodies, specific proteins were detected using goat anti-mouse alkaline phosphatase labeled IgG. Relative expression of MHC class II antigen expression was performed using video densitometry. Positive blots were identified as a band at 30 kD by independent examination by each author.

RESULTS

Fluorescence-activated cell sorting (FACS)

Initial FACS experiments with the rat anti-mouse MHC class I antibody (ATCC TIB 126) generated distinct, predictable fluorescence shifts when assayed either on 3T3 fibroblast cultures (Fig. 1) or on human epidermal cells spiked with 3T3 fibroblasts (Fig. 2). In FACS experiments with CEA cultures, we observed positive results when there was a discernible shift in fluorescence, consistent with the persistence of mouse cells in that experimental culture (Fig. 3). A summary of the FACS results is in Table 1. In primary CEA cultures, we identified 3T3 fibroblasts in 100% (8/8) of cultures. In the secondary and tertiary cultures, we identified 3T3 fibroblasts in 75% (6/8) and 62.5% (5/8) of cultures, respectively. In

addition, we found that percent fibroblast cell survival in CEA ranged from 0.1% to 9.2% and generally decreased with increasing number of CEA passages. We could not obtain complete results for all three culture periods in several patients due to technical problems. Nonetheless, these results indicate that growth-arrested mouse 3T3 fibroblasts can persist long-term in human CEA.

Western Immunoblotting

With evidence suggesting that 3T3 fibroblasts can survive in CEA, we examined whether these cells were immunologically reactive, capable of generating MHC class II antigens in response to treatment with IFN- γ , a potent inducer of MHC class II antigen expression.¹⁸

To ensure that positive immunoblots were due to the expression of mouse specific antigens, we grew CEA either on 3T3 fibroblasts or on LTK⁻ cells, then treated them with or without IFN- γ . As expected, neither LTK⁻ alone nor CEA grown on LTK⁻ generated positive blot results (they should not bind the KL295 class II antibody), confirming the specificity of the antibody for 3T3 MHC class II antigen (Fig. 4). We then performed Western immunoblotting on all of the CEA grown on 3T3 fibroblasts. A summary of these results is in Table 2. Similar to the FACS experiments, we found that mouse MHC class II antigen was present or could be induced in 100% (10/10) of primary cultures, 80% (8/10) of secondary cultures, and 66.7% (6/9) of tertiary cultures. A typical Western immunoblot (patient H) with three positive CEA passages is in Fig. 5. These results demonstrate that persistent 3T3 fibroblasts in CEA can generate MHC class II antigen in response to IFN- γ .

DISCUSSION

In this study, we demonstrate that growth arrested 3T3 mouse fibroblasts

used as feeder layers can persist in multiple passages of CEA. In addition, we demonstrate that 3T3 fibroblast MHC class II antigens persist or can be induced in a majority of CEA cultures.

While the method of generating CEA, initially developed by Rheinwald and Green nearly 20 years ago,^{9, 10} has revolutionized the approach to wound closure particularly in burn patients, a number of investigators have raised concerns about the continued use of CEA. Recent clinical trials report that the results of CEA take are variable and range from 0-80%.² Graft take results in massively burned patients, however, appear to be particularly poor. Recently investigators from the US Army Institute of Surgical Research reported a final graft take of only 32.5% in patients with >70% total body surface area (TBSA) burns whose wounds were excised to fascia and covered with CEA.⁷

A number of predictable factors clearly influence CEA take and include the general medical status of the patient, the status of the pre-grafted wound, the care of the grafted wound, etc. However, there are several intrinsic characteristics of CEA that also effect graft survival. The most obvious of these is graft fragility due to the lack of a dermal component. It has been suggested that the lack of a dermal analog in CEA results in poor anchoring to underlying wounds, increased susceptibility to infection, severe wound contraction, and contributes to graft loss.²

In addition, since the initial clinical trials several years ago, a number of investigators have been concerned about the technique used to generate CEA. Most of this concern has centered on the use of xenogeneic proteins (particularly, FBS) and xenogeneic fibroblast feeder layers (almost exclusively 3T3 Swiss mouse fibroblasts) in CEA cultures.^{3, 11, 12, 19-24} While some of these investigators worry about "contaminating fibroblasts" affecting the biology or functional studies of epidermal grafts,²⁵ others are concerned about the possibility of oncogene transmission.¹¹ Most are concerned, however, about potential xenogeneic antigen

exposure and subsequent activation of the recipient's immune system.^{3, 11, 22}

Our laboratory has previously shown that FBS proteins persist in CEA²⁴ and that patients grafted with CEA generate antibodies to FBS,¹² but these studies did not examine the correlation between CEA survival and elevated anti-FBS levels. While xenogeneic antigen persistence in CEA has historically been considered more of a theoretical, rather than a practical problem, the results of CEA clinical trials, and more specifically, the peculiar pattern of CEA loss in some patients, has renewed interest in this issue. In the study by Rue et.al., six of ten patients with >70% TBSA burn treated with CEA sustained significant graft loss in the absence of demonstrable wound infection, in an immunologic response likened to "rejection".⁷

It is certainly plausible that functional 3T3 fibroblasts might persist in CEA. Though fibroblasts are "lethally irradiated" or "growth arrested" with various agents prior to their use as feeder layers in CEA, 3T3 fibroblasts clearly are still metabolically active, secreting eicosanoids that are thought to contribute to the proliferation and plating of human keratinocytes.²⁶ In addition, as Tolmach and others have demonstrated, a certain percentage of these cells should be expected to survive, metabolically intact, after either one of these "lethal" maneuvers.²⁷⁻²⁹ Finally, while it has been reported that lethally irradiated 3T3 fibroblasts die and float off into the media within 7-10 days of initiating CEA cultures, this phenomenon has never been definitely proven¹¹ and to our knowledge, has not been examined previously by FACS and Western immunoblotting.

In our studies, the FACS and Western immunoblotting data did not always exactly correlate (ie. patients B and H). Nonetheless, in specimens in which both FACS and Western data were available, the data was consistent in 19 of 21 (90.5%) and we found persistent 3T3 fibroblasts in the majority of them (16/21; 76.2%). Though the percentage of cultures with persistent 3T3 dropped off in the tertiary

passages, it was still greater than 50% even in these cultures.

Immune response to antigenic xenogeneic cells intercalated into CEA may produce the multi-focal late graft destruction described by others.^{7,8} Some CEA wounds may heal without late graft loss because xenogeneic fibroblasts do not persist in all CEA, because 3T3 may be destroyed without graft loss, or because the recipient may become tolerant to xenogeneic antigens. Although we have not yet examined the immunologic consequence of 3T3 fibroblast persistence in CEA in this study, we believe the presence of potentially immunogenic 3T3 expressing MHC antigen at any percentage is concerning. This problem could be particularly important for patients who might receive large, repeated applications of CEA for wound coverage (ie. those with massive burn injury). These patients could potentially be at substantial risk for xenogeneic sensitization and the subsequent inflammatory response that results in graft destruction.

In conclusion, 3T3 fibroblasts appear to survive and are immunologically reactive in the majority of CEA examined in this study. The persistence of these mouse fibroblasts may initiate a xenogeneic rejection response that leads to an inflammatory response resulting in CEA loss. While the immunologic significance of this finding has not been proven, the results of these studies suggest that the role of 3T3 fibroblasts in CEA production needs to be re-examined. Ultimately, we believe the long term goal should be the elimination of xenogeneic proteins and feeder layers in the production of clinically available CEA.

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Table 1

Fibroblast Persistence by FACS

| Patient | Culture Passage | | |
|---------|-----------------|-----------|----------|
| | Primary | Secondary | Tertiary |
| A | + (0.70) | + (1.2) | - |
| B | + (1.54) | - | + (1.10) |
| C | + (5.2) | + (2.6) | + (3.1) |
| D | + (0.11) | + (0.39) | + (0.26) |
| E | + (0.13) | + (0.73) | ND |
| F | ND | + (0.11) | + (0.51) |
| G | ND | - | - |
| H | + (9.2) | ND | - |
| I | + (1.4) | ND | ND |
| J | + (2.2) | + (3.4) | + (0.94) |

*Values for FACS probed with anti-murine MHC class I antibody designated as positive (+), negative (-), or not done (ND), with % positive cells in ().

Table 2

Fibroblast Persistence by Western Immunoblot*

| Patient | Culture Passage | | |
|---------|-----------------|-----------|----------|
| | Primary | Secondary | Tertiary |
| A | + | + | - |
| B | + | - | - |
| C | + | + | + |
| D | + | + | + |
| E | + | + | + |
| F | + | + | + |
| G | + | - | - |
| H | + | + | + |
| I | + | + | ND |
| K | + | + | + |

*Values for blots probed with KL295, an anti-murine MHC class II antibody, designated as positive (+), negative (-), or not done (ND).

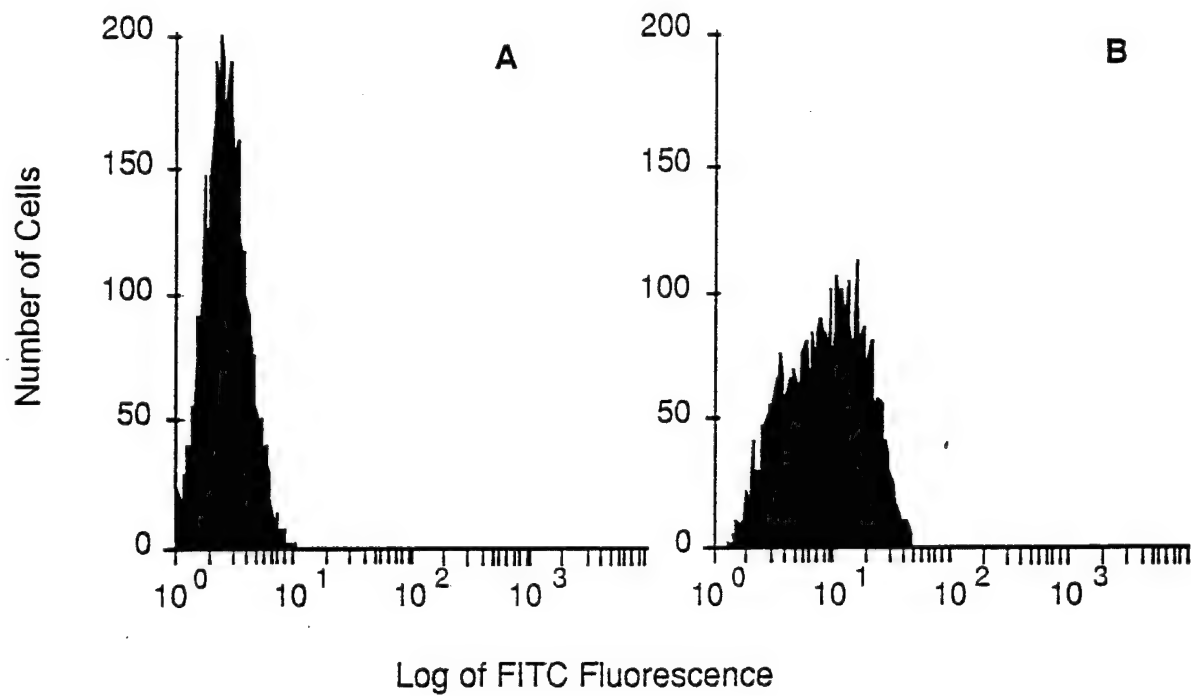
Figure 1. Fluorescence activated cell sorting (FACS) analysis demonstrating shift in fluorescence for 3T3 mouse fibroblasts when analyzed either with irrelevant rat antibody (A) or with rat anti-mouse MHC class I antibody (B).

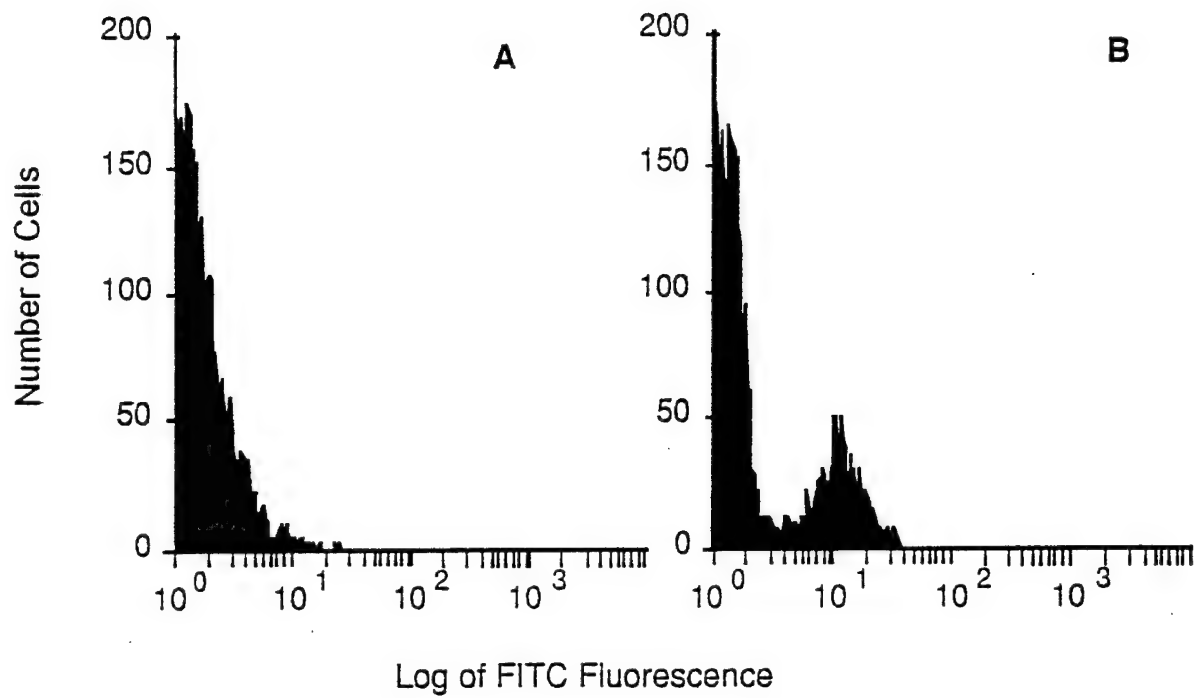
Figure 2. FACS analysis demonstrating a shift in fluorescence for cultured human keratinocytes spiked with a 10% total cell concentration of 3T3 mouse fibroblasts when analyzed either with irrelevant rat antibody (A) or with rat anti-mouse MHC class I antibody (B).

Figure 3. FACS analysis demonstrating a shift in fluorescence for primary epidermal culture (patient C) when analyzed either with irrelevant rat antibody (A) or with rat anti-mouse MHC class I antibody (B). Note the shift in fluorescence that corresponds to the 3T3 fibroblast peak (arrow).

Figure 4. Western immunoblot detection of mouse MHC class II antigen (Ag) in human CEA. 1, positive control for MHC class II Ag; 2a, Primary CEA on LTK⁻ feeder layer (FL); 2b, Primary CEA on LTK⁻ FL treated with interferon-gamma (IFN- γ); 3a Primary CEA on 3T3 FL; 3b Primary CEA on 3T3 FL treated IFN- γ ; 4a, Secondary CEA on LTK⁻ FL; 4b, Secondary CEA on LTK⁻ FL treated with IFN- γ ; 5a Secondary CEA on 3T3 FL; 5b Secondary CEA on 3T3 FL treated IFN- γ . MHC class II Ag only present in 3b and 5b: CEA on 3T3 FL treated with IFN- γ .

Figure 5. Western immunoblot detection of mouse MHC class II Ag in human CEA grown on 3T3 feeder layer (Patient H). 1, positive control for MHC class II Ag; 2, Primary CEA; 3, Second passage CEA; 4, Tertiary passage CEA; 5, negative control.





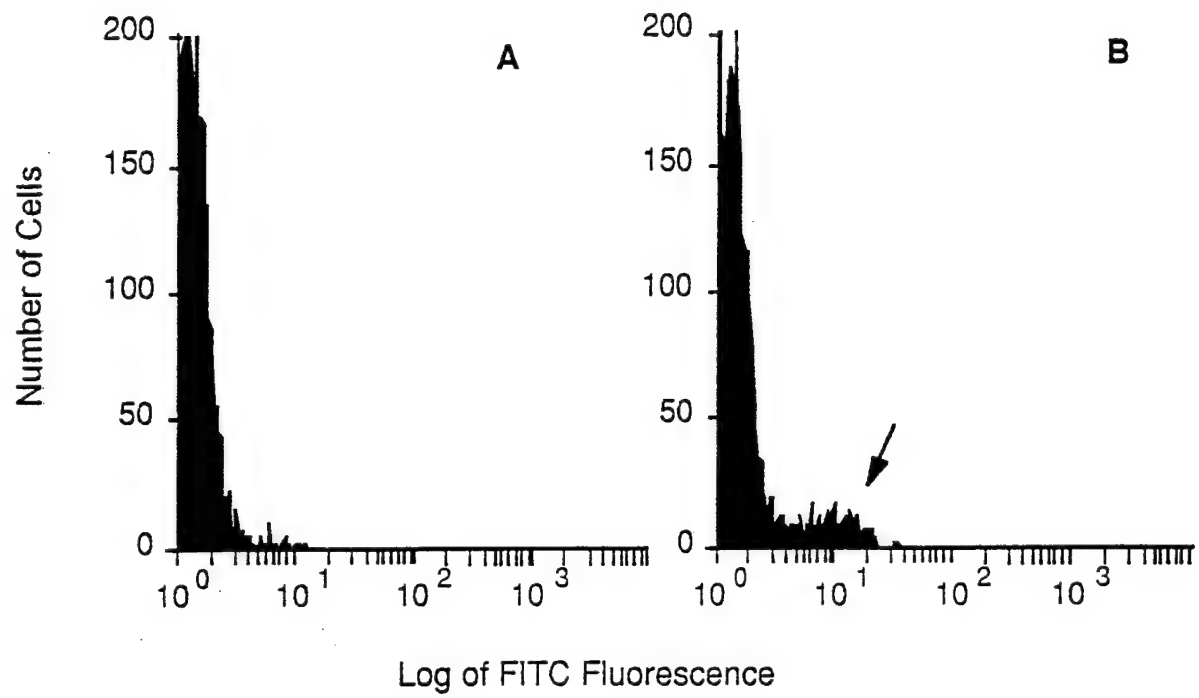


Fig. 4

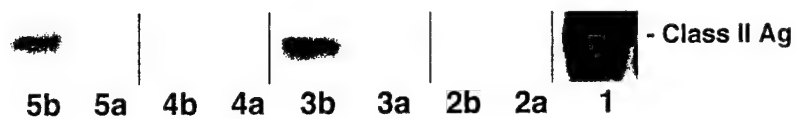


Fig. 5



**Burn Injury Impairs Second Set Rejection and CTL Reactivity
in Mice Primed By Cultured Keratinocyte Allografts**

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ABBREVIATIONS:

| | |
|---------|----------------------------------|
| Allo CK | cultured keratinocyte allograft |
| Allo FT | full-thickness allograft |
| ANOVA | analysis of variance |
| Auto CK | cultured keratinocyte autograft |
| CK | cultured keratinocyte |
| CTL | cytotoxic T lymphocyte |
| FT | full-thickness |
| MHC | major histocompatibility complex |
| TBSA | total body surface area |
| UNC | University of North Carolina |

KEY WORDS: Burn injury, skin replacement, alloantigen, cultured keratinocytes, allograft, autograft, sensitization, priming, cytotoxic T lymphocyte

ABSTRACT

Cultured keratinocyte (CK) allografts have limited antigenicity and have been used as a skin replacement in patients with massive thermal injury. Recent data indicate that CK grafts are more immunogenic than previously believed and could compromise wound healing in the immunocompetent host. The purpose of this study was to determine if the immunosuppression of burn injury might affect the alloantigen response and minimize sensitization to CK allografts. CBA mice (n=146) received a 0%, 20%, or 40% burn that was partially excised three days later and grafted with full thickness (FT) allograft, CK allograft, or CK autograft. Two weeks postburn, mice received FT tail allografts, which were observed for rejection. We observed that FT and CK allografts primed the unburned host with equal efficacy. However, burn injury selectively minimized priming by CK allografts, resulting in delayed rejection of second set allografts.

With evidence that burn injury inhibits host sensitization to CK allografts, we then examined the effect of burn size on CTL alloreactivity. Additional CBA mice (n=36) underwent burn injury, excision, and grafting as described above. Host splenocytes were harvested two weeks later and tested on radio-labeled targets for allospecific cytotoxicity. CTLs from unburned mice primed with FT allografts demonstrated the greatest CTL lysis, followed next by CTLs from unburned mice covered with CK allografts. Burn injury inhibited CTL activity as a function of wound size. Activity of CTLs from burned mice primed with CK allografts improved after in vitro allostimulation but remained below that of CTLs from unburned, unprimed mice. We conclude that burn injury selectively inhibits the allospecific response to CK allografts. The decreased immunogenicity of CK allografts, when used for burn wound coverage, may improve the long-term survival of allogeneic keratinocytes, enhancing their potential as a biologic skin replacement.

INTRODUCTION

Cultured keratinocyte (CK) allografts have been suggested as functional skin replacement in patients with significant thermal injury (1-3), due largely to the limited antigenicity of these grafts. CK allografts are not acutely rejected in burn patients and have extended survival in several animal models, although the long-term fate of these grafts remains debated (4-7). The decreased immunogenicity of CK allografts is based upon the observations that CK allografts do not contain passenger leukocytes (8), do not express MHC class II antigen constitutively (9), and do not elicit an allospecific antibody response or a mixed lymphocyte response in vivo (10).

We have recently reported, however, that CK allografts are more immunogenic than previously believed. In the unburned murine host, CK allografts sensitize for accelerated second set rejection with equal effectiveness as full thickness allografts. This sensitization is associated with an enhanced cytotoxic lymphocyte (CTL) response (11). Keratinocytes can be induced to express MHC Class II antigen when exposed to interferon- γ in vitro (12), and we have demonstrated that CK allografts express substantial MHC Class II antigen within two days of grafting (11).

The clinical significance of these findings is that CK allografts may prime the immunocompetent host and compromise wound healing, thereby limiting the potential of these grafts in wound coverage. Many investigators advocate the use of CK allografts in such dermatologic disorders as epidermolysis bullosa and venous stasis ulcers (13, 14), even though data from randomized, blinded trials are limited. The multiple graft applications required for wound coverage might sensitize these patients and result in chronic inflammation.

The immunosuppression of burn injury may result in impaired priming by CK allografts. Tolerance, or at least non-responsiveness, to CK allografts would improve the utility of this material as a skin replacement and permit burn patients to benefit from this biotechnology. The purpose of this study is to determine if significant burn injury, with its specific defects in cell-mediated immunity, affects host sensitization to CK allografts. We measured alloreactivity of the burned murine host after wound excision and grafting. Sensitization to alloantigens was determined using a model of second set rejection, as well as with assays of CTL function in vitro.

MATERIALS AND METHODS

Experimental Design. The purpose of this study was to determine the effect of burn injury on the immunogenicity of cultured keratinocyte allografts. In the first series of experiments, a model of second set rejection was used to investigate the effect of burn size on CK allograft priming. One-hundred-and-forty-six CBA mice were randomized to receive a 0%, 20%, or 40% total body surface area (TBSA) burn. Seventy-two hours after injury, wounds were partially excised and covered with one of three graft materials: full-thickness C57BL/6 allograft (Allo FT), C57BL/6 allogeneic cultured keratinocytes (Allo CK), or CBA autogenous cultured keratinocytes (Auto CK). Two weeks later, the animals were challenged with full-thickness tail C57BL/6 allografts, which were observed for second set rejection.

In the second series of experiments, we studied the effect of burn injury on CTL alloreactivity to determine if this mechanism was associated with altered priming by CK allografts. Thirty-six CBA mice received a 0%, 20%, or 40% TBSA burn, followed by wound excision 72 hours later and grafting with Allo FT, Allo CK, or Auto FT. Graft

recipient splenocytes were harvested two weeks later, co-cultured with allogeneic stimulators, and used as effectors in subsequent CTL assays.

Animal Protocols. Four- to six-week-old, 15-20 gram, female CBA/J mice (H-2^k) (Harlan Sprague Dawley, Inc., Indianapolis, IN) were used as graft recipients in both experiments. Full-thickness and CK allograft donors were female C57BL/6 mice (H-2^b) (Charles Rivers Laboratories, Wilmington, MA). Animal protocols followed NIH guidelines and had been previously approved by the UNC Committee on Animal Research.

Burn Injury. Mice underwent general anesthesia with methoxyflurane (Pitman-Moore, Washington Crossing, NJ). After being circumferentially clipped, the animals received a full-thickness contact burn, were resuscitated with intraperitoneal Ringer's lactate solution (0.1 ml/g body weight) and subcutaneous morphine sulfate (3µg/g body weight), and were returned to their cages to feed ad libitum. The burn injury was created by applying a 65g brass rod, previously heated to 100°C, to the animal's flank and back for 10 seconds. One application represented 10% of the animal's TBSA, and four anatomically separate applications were necessary to create a 40% TBSA burn. Sham animals receiving the 0% TBSA burn had all of the above interventions, with the exception of the rod application.

Keratinocyte Cultures. Graftable keratinocyte sheets were grown based on the technique originally described by Rheinwald and Green (15). Except where noted, tissue culture materials were obtained from the Lineberger Cancer Center (Chapel Hill, NC).

Three weeks prior to burn injury, donor skin was obtained from C57BL/6 and CBA mice. Full-thickness tail skin was removed, washed in 70% ethanol, and rinsed with Dulbecco's modified MEM (DMEM), supplemented with chloramphenicol and

penicillin/streptomycin. The epidermis was separated from the dermis by incubating the tail skin in 0.25% trypsin (Sigma Chemical Co., St. Louis, MO) for two hours at 37°C. A single cell suspension was created by vortexing the epidermal layer in plating media, which consisted of DMEM and Ham's F-12, 5% Fetal Bovine Serum (Hyclone Laboratories Inc., Logan, UT), insulin 5.0µg /ml, hydrocortisone 0.4µg/ml, transferrin 5.0µg/ml (all from Sigma), cholera enterotoxin 0.01µg/ml (Schwartz Mann/ICN Biomedical Inc., Costa Mesa, CA), 0.1% penicillin/streptomycin, and amphotericin 5.0µg/ml (E. R. Squibb and Sons Inc., Princeton, NJ). Keratinocytes were then co-cultured with a growth-arrested feeder layer of syngeneic murine connective tissue L cells (LTK⁻, H-2^k, ATCC CCL 1.3), previously exposed to mitomycin C 4µg /ml (Sigma) for 45 minutes. LTK⁻ cells and keratinocytes were plated at a density of 2x10⁶ and 4x10⁶ cells/ml, respectively, in plating media that was changed every 2-3 days and supplemented with epidermal growth factor 10.0 ng/ml (Collaborative Research Inc., Bedford, MA) after the first media change. Approximately 10 days after initial plating, LTK⁻ cells were removed by differential trypsinization, and the keratinocytes were allowed to reach confluence.

Wound Excision and Primary Flank Grafting. Three days after burn injury, wounds were partially excised and covered with Allo CK, Auto CK, or Allo FT. Most of the burn wound was left intact to ensure ongoing host immunosuppression. The time course of burn injury, excision, and grafting was chosen to parallel early excision and grafting in humans.

Three weeks after initial plating, CK grafts were gently liberated from culture dishes with dispase (Boehringer Mannheim, Germany), transferred to petroleum-impregnated gauze with the basal surface exposed, and placed directly onto the chest

wall fascia of host CBA mice. Gauze-backed grafts were tucked under the surrounding wound edge and covered with a hydrophilic dressing (Vigilon, C. R. Bond Inc., Berkeley Heights, NJ), which was removed one week later. Allo FT grafts were harvested from C57BL/6 mice, washed in phosphate buffered saline, debrided of subcutaneous fat, and secured to host CBA mice with skin staples. All graft dressings included a circumferential stretch fabric bandage, which was also discontinued after one week.

Second Set Tail Grafting. Two weeks after primary flank grafting, animals were challenged with second set tail grafts to determine the effect of burn injury and CK allografting on host sensitization. The tails of anesthetized mice were washed with ethanol and grafted, via a method previously described (16), with a 10 mm full-thickness skin allograft (C57BL/6) placed on the dorsal surface of the tail. Animals also received a 10 mm full-thickness autograft (CBA), which served as an internal control. Specially manufactured cylindrical glass tubes were used to protect the tail grafts from mechanical disruption and organic debris. The tubes were removed three days later, and the tail grafts were assessed daily for viability by two independent observers. Objective evidence of rejection was based on criteria of graft color, hair orientation, and scale integrity. Median tail allograft survival time for each of the nine groups was then determined and compared for statistical significance.

CTL Alloreactivity. To determine if specific defects in CTL function might account for altered sensitization after burn injury, we repeated the above experiment but examined CTL alloreactivity in the place of second set rejection. Two weeks after burn injury and grafting with Allo FT, Allo CK, and Auto FT, CBA hosts were sacrificed and splenocytes were harvested for CTL assays. Graft recipient splenocytes (2×10^6 cells/ml, CBA, mice H-2^k) were mixed with irradiated (2000 rads) donor splenocytes

(2×10^6 cells/ml, C57BL/6, H-2^b) in RPMI 1640 media containing 10% FBS, 0.1% penicillin/streptomycin, and 5×10^{-5} M 2-ME (Sigma). These two groups were co-cultured in 24 well plates, under standard incubator conditions of 5% CO₂ and 37°C.

After 0, 3, 4, 5, 6, and 7 days of in vitro stimulation, CTL alloreactivity was assessed by isolating and washing effector lymphocytes, and testing these cells on both positive targets (EL-4 murine lymphoma cells, H-2^b, ATCC TIB 39) and negative targets (LTK⁻, H-2^k, ATCC CCL 1.3), which had been preloaded with 100 μ Ci of Na₂⁵¹CrO₄ (ICN Biomedicals Inc., Irvine, CA) for 30 minutes. Targets were placed in round-bottom 96 well plates (1×10^6 cells/well), to which varied numbers of CTL effectors (1.25×10^5 - 1.5×10^6 cells/well) were added, in a volume of 150 μ l of RPMI 1640 with 5% FBS. Following four hours of incubation at 37°C, target lysis was assessed by measuring the release of Na₂⁵¹CrO₄ into the media. Supernatants were collected and measured for radioactivity with a γ -counter. Each condition was tested in duplicate, with radioactivity expressed as mean cpm.

Specific cell lysis was determined by comparing radioactivity of the samples with spontaneous release of radioactivity from solitary targets, as well as maximum release of radioactivity from targets lysed by 5% Triton X-100 detergent. The following formula was used to calculate specific CTL killing: % specific lysis = $[(\text{cpm}_{\text{sample}} - \text{cpm}_{\text{spontaneous}}) / (\text{cpm}_{\text{maximum}} - \text{cpm}_{\text{spontaneous}})]$. Average spontaneous target release was less than 10% of the potential maximum release. This experiment, which included six CTL assays (performed after 0, 3, 4, 5, 6, and 7 days of in vitro stimulation), was conducted three times with similar results.

Statistical Analysis. Median tail allograft survival times were compared between groups by Wilcoxon rank and χ -square analysis. CTL alloreactivity, as

measured by % specific lysis, was compared between groups by two-way ANOVA. Statistical significance was defined at $p < 0.05$.

RESULTS

Animal Survival. The full-thickness contact burn model described in these and other experiments produces predictable mortality that presumably corresponds with the degree of host immunosuppression. In general, the sham burn produces no mortality, a 20%TBSA burn has 10% mortality, and a 40% burn yields 40% mortality. No animal deaths occurred during the early post-burn period (<48 hours), as a result of inadequate resuscitation, or in the late post-burn period (>2 weeks), after flank wounds were completely healed. Those deaths which did occur resulted from either anesthetic complications or generalized sepsis. Autopsies performed on the nonsurviving animals demonstrated bowel edema and dilatation, consistent with ileus and partial obstruction. No obvious wound infections were observed, although it is assumed that burn wounds were colonized with bacteria.

Second Set Rejection. To determine the effect of burn size on the immunogenicity of allogeneic cultured keratinocytes, we utilized a model of second set rejection, as originally described by Medawar (17). Animals pre-exposed to alloantigen will mount a more vigorous rejection response when re-exposed to that particular antigen. We previously reported that both Allo CK and Allo FT prime unburned mice with equal efficacy (11), despite the fact that Allo CK grafts lack passenger leukocytes and do not constitutively express MHC Class II antigen (8, 9). However, the effect of burn injury on allo-sensitization was unknown.

In the first experiment, animals received a 0%, 20%, or 40% TBSA burn injury, followed by partial wound excision and grafting with Allo CK, Allo FT, or Auto FT. Two weeks later, animals underwent second-set tail grafting. Median tail graft survival times were determined for each of the nine groups and compared for statistical significance. The results are listed in Table 1. As noted previously, unburned mice primed with Allo CK and Allo FT rejected tail allografts after nine days, compared to tail allograft survival of 13 days in mice who were not primed ($p < 0.01$).

Burn injury, however, significantly extended second set allograft survival in all three grafting groups. A 40% TBSA burn prolonged unprimed tail graft survival from 13 to 15 days ($p < 0.01$). In mice primed with Allo CK, 40% burn prolonged tail graft survival from 9 to 14 days ($p < 0.01$), compared to 12.5 days in mice primed with Allo FT ($p < 0.01$). Therefore, burn injury impairs priming by Allo CK to a greater extent than priming by Allo FT ($p < 0.05$). This difference in host sensitization to alloantigen is more apparent as burn size increases. Median tail graft survival was not statistically different for mice receiving a 40% burn and grafted with either Auto CK or Allo CK ($p = 0.07$). A 20% TBSA blunted but did not abolish Allo CK priming, whereas the 40% burn almost eliminated priming, as determined by second set rejection.

The results of the second set rejection experiments can also be seen comparing tail graft survival curves. Figure 1 shows the effect of burn size on priming by Allo CK. Positive and negative controls include tail graft survival curves from unburned mice who were primed with either Allo CK or Auto CK, respectively. Animals exposed to alloantigen from FT grafts are represented by solid circles, while mice grafted with Allo CK are represented by open circles. Tail graft survival curves from mice who were burned are represented by dashed lines. Burn injury inhibits second set rejection (or

prolongs tail graft survival), resulting in a shift of the tail graft survival curves to the right. Burn injury, therefore, impairs the sensitizing effects of Allo CK, and the magnitude of impairment increases as burn size increases.

CTL Alloreactivity. With evidence that burn injury inhibits second set rejection, we asked if this impaired priming might correspond with specific defects in CTL function. CTL alloreactivity after burn injury and grafting was determined by stimulating graft recipient splenocytes with alloantigen in vitro for 0-7 days. To characterize the generation of alloreactivity and to describe the kinetics of the response, CTL function was assessed at various time points after stimulation. Effector lymphocytes were exposed to radiolabelled targets, and specific cell lysis was used as an index of CTL alloreactivity. The effect of burn injury and grafting on CTL response, over several days, can be seen in Figures 2-4.

No CTL activity was observed initially (data not shown). After three days of allostimulation, only unburned mice primed with Allo FT had any substantial CTL activity (Figure 2). By day four, however, CTL function increased for several of the groups (Figure 3). The greatest CTL activity came from mice grafted with Allo FT. This is followed next by CTLs from animals grafted with Allo CK, which is slightly greater than CTLs from unburned, naive mice.

Burn injury serves to blunt the CTL response in animals primed with Allo CK, which is consistent with the results of the second set rejection experiment. This decrease in CTL alloreactivity, as measured by % specific lysis, is dependent on the size of the burn (Figure 3). After seven days of in vitro stimulation, however, CTL alloreactivity from these burned mice improves but remains significantly below that of baseline CTL activity from unburned, unprimed controls (Figure 4). CTL function from

unburned mice primed with Allo FT or Allo CK is still greater than that of CTLs from unburned, unprimed mice.

DISCUSSION

In this series of experiments, we extend our previous findings that CK allografts and FT allografts prime the unburned host with equivalent efficacy. We provide evidence that burn injury selectively impairs sensitization to CK allografts. Specifically, burn injury inhibits second set rejection as a function of burn size, with a greater effect on priming by CK allografts, compared to FT allografts. This defect in antigen processing can also be observed in the effect of burn injury on CTL alloreactivity. Burn injury decreases CTL responsiveness to grafted, allogeneic keratinocytes. This impaired CTL alloreactivity improves after several days of *in vitro* stimulation, but fails to return to baseline levels of CTLs not previously exposed to alloantigen.

Specific defects in CTL function may contribute to the prolonged survival of CK allografts after burn injury. Initial enthusiasm for the use of these grafts as a biologic skin replacement came from the theoretical belief that CK allografts, which are devoid of Langerhans cells and do not constitutively express MHC Class II antigen, were non-immunogenic and might survive permanently (8, 9). This putative lack of antigenicity results in the inability of keratinocytes to stimulate allogeneic lymphocytes *in vitro* (18), and allows for CK allografts to survive apparently indefinitely in several rodent models, including mice presensitized with FT allografts (19). These results encouraged clinical trials with CK allografts, with the supposition that allogeneic keratinocytes might not be rejected and might function as a permanent skin replacement.

CK allografts were first used for burn wound coverage in 1983, when Hefton and Shires reported the success of these grafts in three patients who survived deep partial-thickness burn injuries (1). Several case reports and patient series validated the utility of CK allografts in providing wound coverage, confirmed the fact that these grafts were not acutely rejected, and suggested that they might persist indefinitely (2, 3, 6). However, investigators discovered that CK allografts placed on partial thickness wounds were gradually replaced by host keratinocytes, as determined by DNA fingerprinting (20) and cytokeratin analysis (21). In partial thickness burns tangentially excised and covered with CK allografts, residual dermal elements such as hair follicles and sweat glands presumably repopulate the wound and replace donor-specific keratinocytes with host keratinocytes. The fate of CK allografts when placed on fascia or granulation tissue remains unknown.

Our recent report that CK allografts prime the unburned host and express MHC Class II antigen in vivo (11) raises concern that multiple applications of these grafts might be counterproductive to wound healing by creating a state of chronic inflammation. Although two blinded, prospective studies have demonstrated improved wound healing in donor sites covered with CK allografts, the subjects in these studies had a single, limited application of allogeneic keratinocytes and are not representative of patients requiring a large area of excision and coverage (22, 23). Additionally, the unpredictable take of cultured keratinocytes, which ranges from 20-80%, obligates many patients to receive more than one application of grafts (24).

Nevertheless, the immunosuppression of burn injury appears to interfere with antigen processing and host responsiveness to CK allografts, thereby improving the utility of these grafts in long-term wound coverage. Burn injury has been noted to

delay rejection of cadaveric skin allografts, as well as increase host susceptibility to viral infection (25). Cyclosporin, which is a potent inhibitor of IL-2 production, has been used experimentally (26) and clinically (27) to induce cadaveric skin non-responsiveness, although long-term graft survival has not been possible. CK allografts, on the other hand, are significantly less antigenic than full-thickness allografts and do not sensitize the burned host as well as the immunocompetent host. Although some priming does occur after a 20% TBSA burn injury, the immunosuppression of a 40% injury nearly eliminates sensitization, as measured by second set rejection. Patients with much larger burns (70-95% TBSA) would be those individuals who need CK allografts and may be effectively unresponsive to these allogeneic cells. Further understanding and manipulating the mechanism of this impaired priming may permit graft tolerance, or at least non-responsiveness, which would allow the use of CK allografts in early, permanent burn wound coverage.

A possible mechanism for altered responsiveness to CK allografts may be related to the effect of burn injury on CTL function. CTLs play a critical role in the effector arm of allograft recognition and rejection (28). Combined with specific defects in IL-2 transcription and helper cell function (29), decreased CTL alloreactivity after burn injury helps to explain CK allograft non-responsiveness and is consistent with the impaired second set rejection already described. How burn injury impairs the CTL response remains speculative, but could include sequestration of CTLs in the burn wound, impaired function of available CTLs, or inhibition of precursor CTL populations. The observation that CTL alloreactivity improves after *in vitro* stimulation with alloantigen, but does not return to normal, suggests an intrinsic, functional defect in target-specific killing.

In summary, CK allografts and FT allografts prime the unburned host for accelerated second set rejection and enhanced CTL alloreactivity. Burn injury, with its associated defects in cell-mediated immunity, impairs alloantigen sensitization, especially as burn size increases. Although CK allografts are more immunogenic than previously believed, burn injury selectively inhibits sensitization to CK allografts, compared to FT allografts, with the greatest difference in priming observed after a 40% burn. This decreased immunogenicity of CK allografts, in the clinical situation of burn wound coverage, may improve the long-term survival of allogeneic keratinocytes and enhance their potential as a biologic skin replacement.

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TABLES

Table 1. Second Set Tail Allograft Rejection.

| Burn Size | Median Tail Graft Survival, days (range) | | |
|-----------|--|---------------------|----------------------|
| | Primary Flank Graft | | |
| TBSA | Auto CK | Allo CK | Allo FT |
| 0% | 13 (10-15) n=15 | 9 (7-11) n=15 | 9 (7-11) n=18 |
| 20% | 14 (12-17) n=17 | 11.5 (9-15) n=18 | 10 (8-12) n=16 |
| 40% | 15 (13-18) n=13 | 14 (10-16) n=18 | 12.5 (10-16) n=16 |

FIGURES

Figure 1. Survival curves of secondary tail allografts, depicting the effect of burn injury on second set rejection. Both Allo FT and Allo CK primed the unburned host with equal efficacy. Burn injury significantly inhibited sensitization by Allo CK, as evidenced by prolonged survival of second set tail allografts.

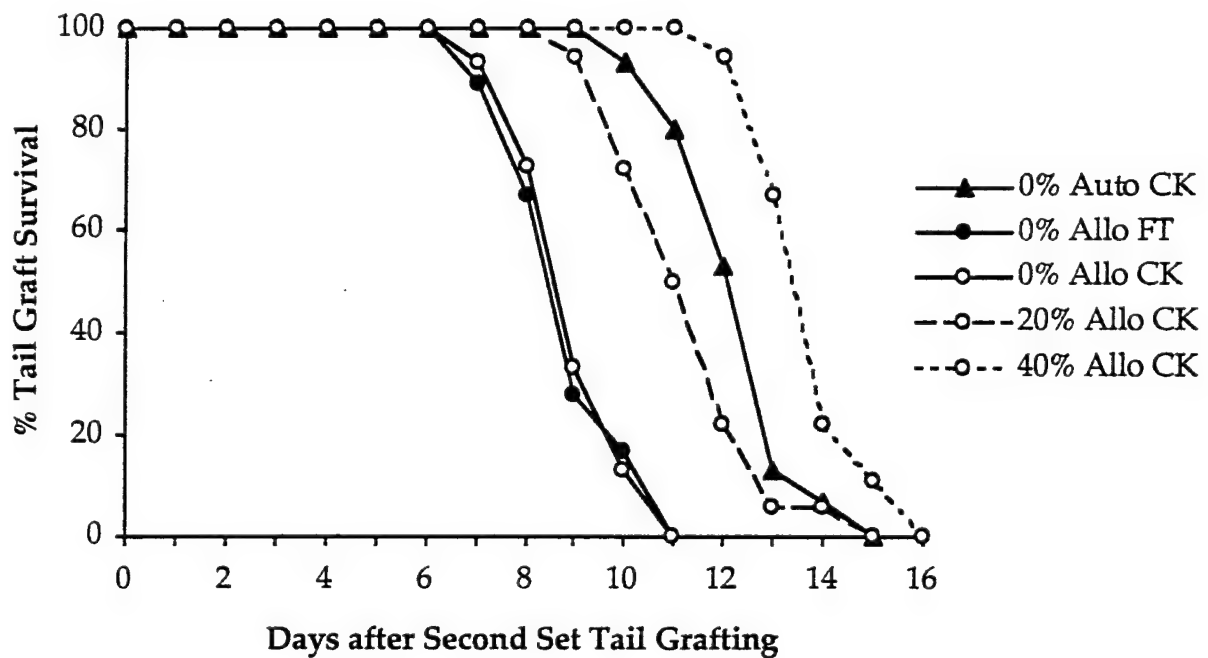


Figure 2. The effect of burn injury on CTL function, after 3 days of in vitro allostimulation.

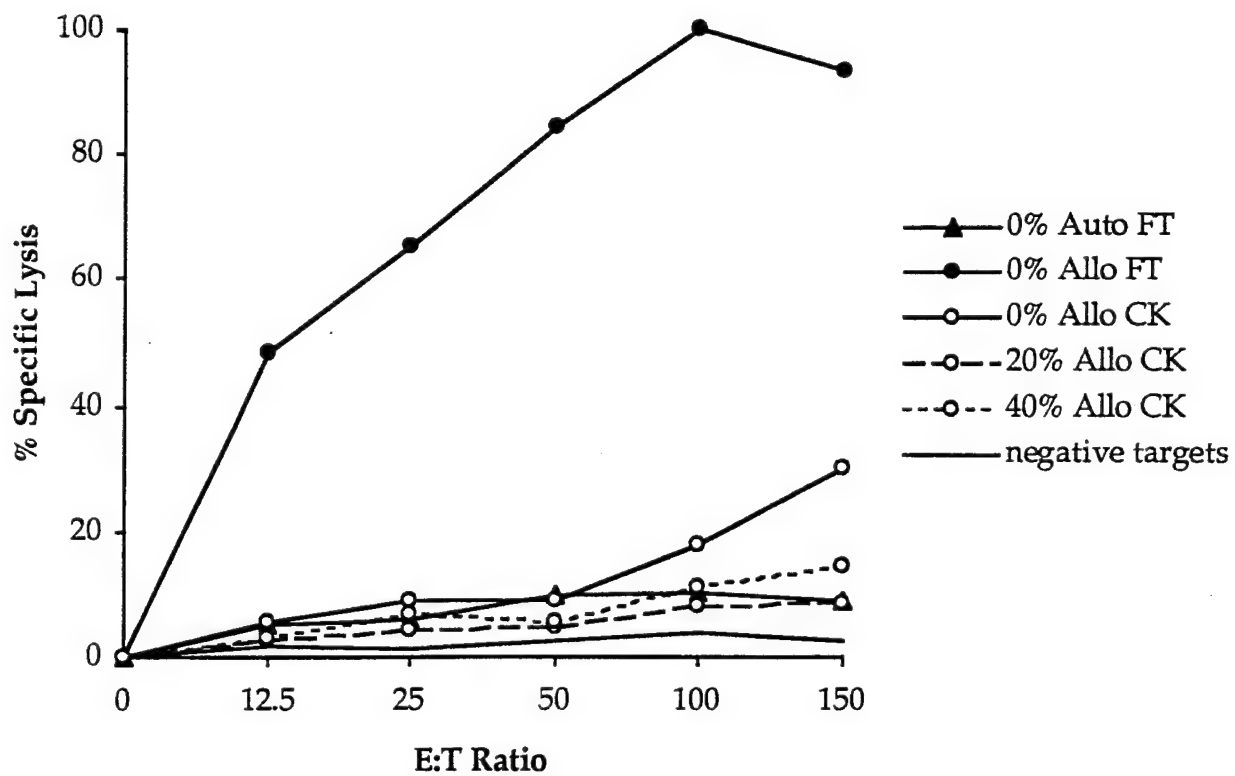


Figure 3. The effect of burn injury on CTL function, after 4 days of in vitro allostimulation.

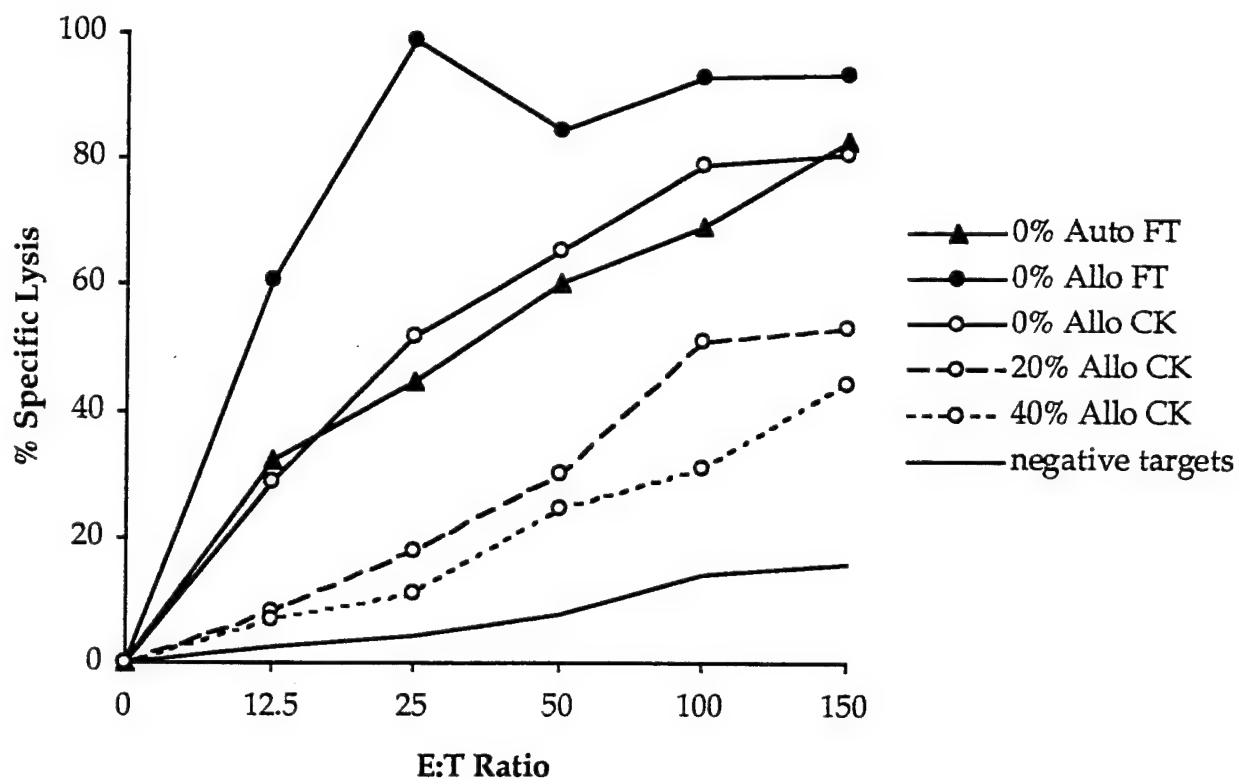


Figure 4. The effect of burn injury on CTL function, after 7 days of in vitro allostimulation.

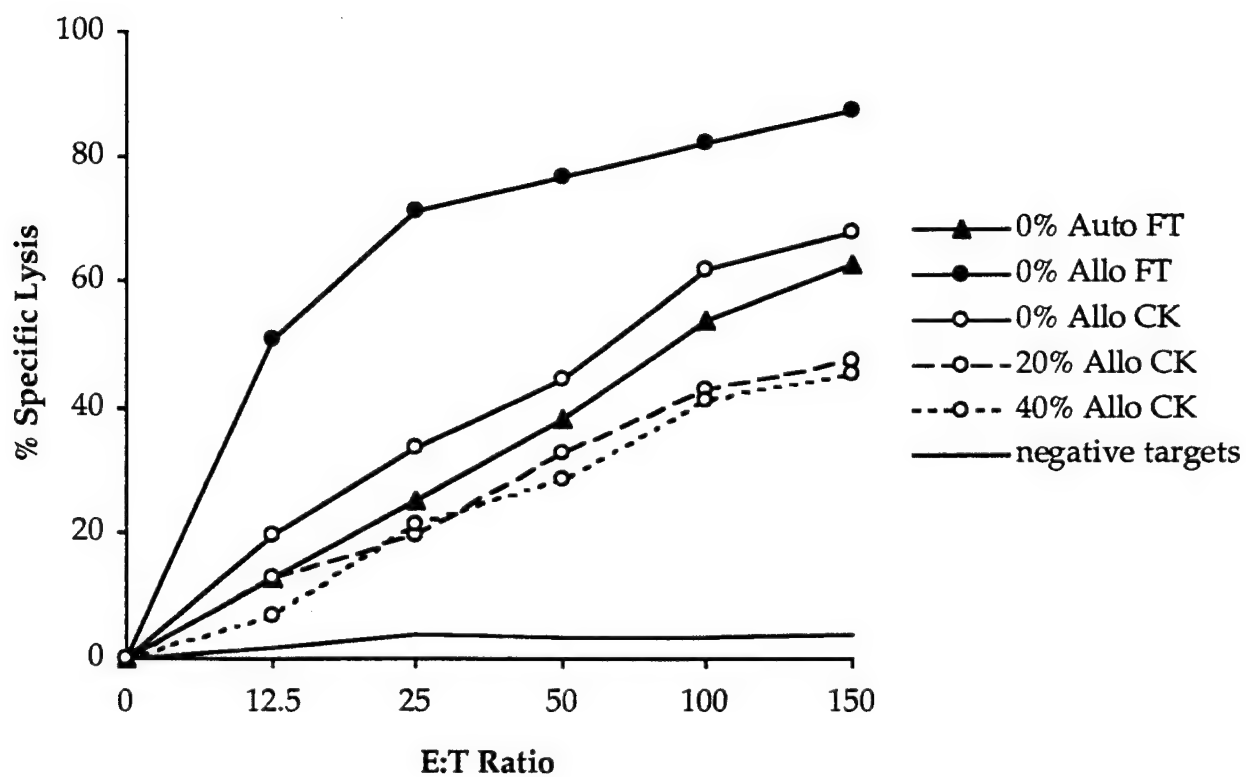


FIGURE LEGENDS

Figure 1. Survival curves of secondary tail allografts, depicting the effect of burn injury on second set rejection. Both Allo FT and Allo CK primed the unburned host with equal efficacy. Burn injury significantly inhibited sensitization by Allo CK, as evidenced by prolonged survival of second set tail allografts.

Figure 2. The effect of burn injury on CTL function, after 3 days of in vitro allostimulation

Figure 3. The effect of burn injury on CTL function, after 4 days of in vitro allostimulation

Figure 4. The effect of burn injury on CTL function, after 7 days of in vitro allostimulation

Early, Complete Burn Wound Excision Partially Restores Cytotoxic T Lymphocyte Function

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RUNNING TITLE: Burn wound excision and CTL function

KEY WORDS: Burn injury, wound excision, cytotoxic T lymphocyte, immunosuppression, alloreactivity, alloantigen

ABSTRACT

Background: Cytotoxic lymphocytes (CTLs) are an important component of immune function, involved in antigen recognition and resistance to viral infection. Burn injury suppresses cell-mediated immunity, induces allograft tolerance, and increases the risk of viral infection, but the mechanisms are not well understood. This study analyzes the effect of burn size and burn wound excision on CTL activity.

Methods: Anesthetized CBA mice (n=12) received a 0%, 20%, or 40% body surface area contact burn. Additional mice (n=16) received a 40% burn that was totally, partially, or not excised 72 hours post-burn. Excised areas were covered with normal, syngeneic skin. Two weeks later, harvested splenocytes were co-cultured with allogeneic stimulators. CTL activity was determined by a ^{51}Cr release assay, in which CTL effectors were tested on allogeneic, radio-labeled targets. Dilution curves of CTL activity were compared by ANOVA.

Results: Both 20% and 40% burns significantly inhibited CTL activity ($p < 0.05$). Total but not partial excision of a 40% burn restored CTL activity ($p < 0.01$). Both total and partial wound excision also improved survival ($p < 0.05$).

Conclusion: Burn injury inhibits CTL activity in a size-dependent manner, and total wound excision significantly improves both CTL function and survival after injury. This study suggests a mechanism for the immunosuppressive effects of burn injury and provides an immunologic rationale for early, complete burn wound excision.

INTRODUCTION

Thermal injury initiates a cascade of physiologic changes which ultimately result in host immunosuppression. While specific defects in both humoral and cellular immunity have been demonstrated, the effect of burn injury on T cell function has not been completely described. Burn injury has been noted to delay allograft rejection,¹ to decrease contact hypersensitivity,² and to increase susceptibility to viral infection.³ This suggests that thermal injury may inhibit the activity of cytotoxic T lymphocytes (CTLs), important effector cells involved in antigen recognition, processing, and elimination.

Early, sequential burn wound excision has become the standard of care in the management of significant thermal injury. Such therapy is partially based on the supposition that continued presence of burn eschar perpetuates host immunosuppression. Many clinical studies support the concept of early burn wound excision⁴⁻⁷ and demonstrate improved survival,^{4,5} decreased length of hospitalization,⁶ and less hypertrophic scarring⁶ compared to conservative therapy. However, only selected groups of patients clearly benefit from early excision, such as children^{4,5} and individuals without inhalation injury.⁵

Whether or not early burn wound excision decreases infectious complications remains controversial. Few studies have actually examined the role of eschar excision in correcting lymphocyte dysfunction after thermal injury. Nevertheless, the immunologic benefits of early wound excision and closure do include decreased release of pro-inflammatory mediators,⁸ decreased endotoxemia,⁹ and restoration of contact hypersensitivity.¹⁰ Furthermore, transfer of burn eschar to the unburned host inhibits cell-mediated immunity, as measured by lymphocyte mitogenesis and expression of cell surface antigens.¹¹ However, the cellular mechanisms for these effects remain elusive.

In this experiment, we studied the effect of burn size and burn wound excision on CTL function. Specifically, we asked if the degree of immunosuppression, as measured by

CTL alloreactivity, was related to the surface area of the burn eschar. We hypothesized that elimination of the burn wound, if performed early after injury, would restore host immunocompetence. Improved CTL function would provide an immunologic rationale for early burn wound excision and, consequently, might decrease the incidence of post-burn infection, which remains the leading cause of death following thermal injury.⁷

MATERIALS AND METHODS

Experimental Design. In the first experiment, CBA mice (n=12) were randomized to receive a 0%, 20%, or 40% total body surface area (TBSA) burn, which was partially excised and covered with syngeneic skin three days later. Two weeks after burn injury, host splenocytes were harvested, stimulated with growth-arrested allogeneic splenocytes, and used as CTL effectors after 0, 3, 4, 5, 6, and 7 days of in vitro stimulation. The second experiment investigated whether or not early burn wound excision might restore host immunocompetence. CBA mice (n=16) received a 40% TBSA burn which was completely, partially, or not excised three days later and covered with normal, syngeneic skin. Splenocytes were collected two weeks after burn injury, co-cultured with allogeneic stimulators, and used as effectors in subsequent CTL assays.

Animal Protocols. Fifteen to twenty gram, four- to eight-week-old inbred female CBA/J mice (H-2^k) (Harlan Sprague Dawley, Inc., Indianapolis, IN) were used as subjects in both experiments. Syngeneic skin for wound coverage was obtained from unburned CBA mice of equal age and weight. Allogeneic splenocytes from female C57BL/6 mice (H-2^b) (Charles Rivers Laboratories, Wilmington, MA) were used as stimulators in the CTL assays. Animals were cared for in accordance with NIH guidelines, and all protocols had previously been approved by the UNC Committee on Animal Research.

Burn Injury. After induction of general anesthesia with methoxyflurane (Pitman-Moore, Washington Crossing, NJ), animals were circumferentially clipped and washed. A full-thickness contact burn was created by applying a 65g copper rod, previously heated to 100°C, to the animal's back and/or flank for 10 seconds. One application represented 10% of the animal's TBSA, with four individual applications necessary to create a 40% TBSA burn. In addition to producing predictable mortality, the contact burn used in this model yields an injury of consistent depth and identifiable border, which permits precise wound excision and closure. Survival following a 40% full-thickness contact burn is typically 65%, while survival after a 20% burn is 90%.

Following burn injury, mice were resuscitated with intraperitoneal lactated Ringer's solution (0.1 ml/g body weight) and were given subcutaneous morphine sulfate (3µg/g body weight) for postburn pain control. Animals were returned to individual cages and allowed to feed ad libitum. Sham animals with the 0%TBSA burn underwent all of the described interventions, except for the actual burn injury.

Wound Excision and Closure. The time course of burn injury, wound excision, and grafting was chosen to approximate the clinical course of these parameters in human burn patients. In the first experiment, a limited portion of the flank wound (representing 10% TBSA) was excised three days after injury, leaving a significant amount of eschar intact to ensure continued host immunosuppression. Wounds in each burn group (0%, 20%, and 40%) were excised via sharp dissection to the level of musculoskeletal fascia. Full-thickness autografts, previously debrided of adipose and rinsed in phosphate buffered saline, were placed directly onto fascia, secured to the surrounding wound edges with skin staples, and protected with a circumferential bandage that was removed after one week.

In the second experiment, mice receiving a 40% contact burn underwent total, partial, or no excision three days after burn injury. Wounds were covered with normal, syngeneic skin as described above. "Total excision" involved complete removal of the burn eschar, "partial excision" involved removal of 10% of the 40% TBSA burn, and "no excision" involved leaving the burn wound undisturbed. Mice in the "unburned sham" group received general anesthesia and resuscitation, but not burn injury or excision.

CTL Assays. Postburn CTL activity was assessed two weeks after injury, to coincide with the maximum predicted suppression in lymphocyte effector function suggested by other reports.¹²⁻¹⁴ Harvested splenocytes were washed three times in growth media (RPMI 1640, 10% FBS, 0.1% penicillin/streptomycin, 5×10^{-5} M 2-ME; obtained from the Lineberger Cancer Center, Chapel Hill, NC), assessed for viability via trypan blue exclusion, and co-cultured with growth-arrested (2000 rads) allogeneic splenocytes in standard incubator conditions of 37°C and 5% CO₂.

After 0, 3, 4, 5, 6, and 7 days of in vitro stimulation, CTL alloreactivity was assessed by exposing effector lymphocytes to potentially recognizable positive targets (EL-4 murine lymphoma cells, H-2^b, ATCC TIB 39), as well as MHC-identical negative targets (LTK⁻, H-2^k, ATCC CCL 1.3), at effector to target (E:T) ratios of 0, 12.5, 25, 50, 100, and 150. Prior to exposure, targets had been preloaded with 100 μ Ci of ⁵¹Cr (ICN Biomedicals Inc., Irvine, CA). After a 4 hour, 37°C incubation, CTL activity against these targets was determined by measuring the release of ⁵¹Cr from target cells into the media. Supernatants were collected and quantified for radioactivity, expressed as mean cpm. CTL killing was determined by the formula: % specific lysis = $100 * [(cpm_{\text{sample}} - cpm_{\text{spontaneous}}) / (cpm_{\text{maximum}} - cpm_{\text{spontaneous}})]$. A total of 24 assays were performed, with each condition tested in duplicate.

Statistical Analysis. CTL activity, as determined by % specific lysis, was compared between groups by multi-factor ANOVA with replication. Differences in postburn

survival were tested by χ -square and corrected for multiple comparisons. Differences in postburn weight were analyzed by ANOVA. Statistical significance was defined at $p < 0.05$.

RESULTS

Animal Survival. Separately performed mortality studies involving 38 CBA mice receiving a 40% burn demonstrated significantly improved survival following either total or partial wound excision 72 hours after injury ($p < 0.05$, χ sum=9.30, Table). Survival was 100% after sham burn, 100% after total excision, 90% after partial excision, and 58% after no excision. In contrast to these benefits, neither total nor partial wound excision significantly mitigated postburn weight loss. Percent change in body weight, 14 days postburn, was 104% after sham injury, 101% after total excision, 99% after partial excision, and 95% after no excision. While excision slightly improved postburn weight gain, compared to the unexcised group, this difference was not statistically significant ($p = 0.08$).

Effect of Burn Size on CTL Function. To determine the effect of burn size on CTL function, we subjected mice to a 0%, 20%, or 40% burn that was partially excised, leaving a substantial amount of eschar intact to ensure continued host immunosuppression. Two weeks after injury, harvested splenocytes were stimulated with allogeneic, growth-arrested splenocytes. Such stimulation induces CTL proliferation and results in the generation of an allospecific response over time. Primed CTLs will carry out perforin-dependent, MHC I-restricted cell lysis when re-exposed to alloantigen. In this study, CTLs were tested on radiolabeled allogeneic targets 0, 3, 4, 5, 6, and 7 days after stimulation, with cell lysis used as an index of CTL function.

Without allostimulation, on day 0 of the assay, CTL activity against all targets was negligible (data not shown). After 4 days of stimulation, CTLs from unburned mice

demonstrated considerable activity against positive targets, but CTLs from mice receiving either a 20% or 40% burn did not lyse these targets as efficiently ($p < 0.05$, Figure 1). The greatest defect in CTL function was observed in the 40% burn group, which failed to demonstrate any activity against positive targets, compared to non-specific lysis of negative targets ($p < 0.01$). Continued in vitro stimulation, after 5 days, improved CTL function in the 0% and 20% burn groups, but CTL activity from the 40% burn group remained significantly depressed ($p < 0.01$, Figure 2). This defect in MHC-specific target lysis persisted throughout the course of the experiment and remained uncorrected despite continued in vitro stimulation. After 6 days of stimulation with alloantigen, CTL function approached normal for the 20% burn group and improved considerably for the 40% burn group (Figure 3). Nevertheless, CTL activity remained significantly depressed for both burn groups ($p < 0.05$). In summary, both 20% and 40% burn wounds suppressed CTL activity, with the 40% burn injury producing a more pronounced and longer lasting defect in CTL function, which remained refractory to allostimulation.

Effect of Burn Wound Excision on CTL Activity. With evidence that a partially excised 40% TBSA burn inhibits CTL activity, we hypothesized that wound excision might improve CTL function. After receiving a 40% burn, mice underwent total, partial, or no wound excision 72 hours later. Two weeks postburn, harvested splenocytes were co-cultured with allogeneic stimulators and subsequently used in CTL assays. No CTL activity was observed in unstimulated splenocytes (data not shown). After 3 days of allostimulation, minimal but significant lysis of positive targets was noted by CTLs from the unburned controls, as well as from animals who had complete wound excision ($p < 0.05$, Figure 4). These differences, however, were not significant at every E:T ratio but were significant by multi-factor ANOVA.

Following 4 days of stimulation, CTL activity increased dramatically for the sham burn group, followed next by CTL activity from mice whose wounds were completely excised (Figure 5). CTL function in the partial or no excision groups improved but displayed significantly less activity than that of the sham burn or total excision groups ($p < 0.01$). CTLs from the unexcised group displayed slightly greater killing than CTLs from the partially excised group, but this difference was not significant. Continued stimulation after 5 days produced similar results (Figure 6). Improved CTL function in the total excision group, relative to the partial or no excision groups, was not observed on day 6 of stimulation (Figure 7) but returned on the seventh and final day of the assay (Figure 8). Overall CTL killing diminished, but both the unburned sham and total excision groups displayed significantly greater CTL activity than the partial or no excision groups ($p < 0.01$).

In summary, total excision of a 40% burn wound improved both survival and CTL effector function. However, only on day 3 of allostimulation did total wound excision completely restore CTL activity. Partial wound excision did improve survival but did not confer the gains in CTL function that total excision did. For the partial excision group, CTL responsiveness to alloantigen improved after in vitro stimulation, but remained equivalent to CTL activity in the group whose burns were left undisturbed.

DISCUSSION

In this study, we demonstrate that thermal injury inhibits CTL activity as a function of burn size. Both 20% and 40% TBSA full-thickness burns, although partially excised, were associated with significant CTL dysfunction 14 days after injury. The ability of CTLs from burned mice to recognize and lyse allogeneic targets improved after in vitro stimulation, but remained depressed when compared CTLs from unburned

controls. Such defects in CTL activity may represent decreased numbers of CTLs (or CTL precursors) within available lymphocyte compartments, decreased responsiveness to allogeneic stimulation, or a long-lasting impairment in CTL effector function.

The immunosuppression of thermal injury has been extensively studied and consists of well-described defects in humoral and cellular immunity. Specific changes in T cell immunity include the altered distribution of lymphocyte subpopulations,^{15,16} the emergence of suppressor T cells,¹⁷ decreased production of IL-2,¹⁴ and decreased response to mitogens.¹⁴ Such disruption of host immunocompetence contributes to increased susceptibility to bacterial and viral infection,³ delayed allograft rejection,¹ and decreased responsiveness to cutaneous hypersensitivity antigens.^{2,10}

A limited number of experimental studies have demonstrated defects in the effector function of T lymphocytes. Using a 66% TBSA burn, Markley and Smallman observed that mice sensitized to alloantigen before and after injury had decreased lymphocyte cytotoxicity, as measured by the ⁵¹Cr release assay.¹² Klimpel et al reported that naïve natural killer (NK) cells from thermally injured humans had decreased activity against K562 tumor cells and HSV-1 infected Raji tumor cells. Pretreatment of NK cells with IL-2 or IFN- α improved effector function only in unburned controls and in patients who did not have severely depressed lymphocyte cytotoxicity.¹³ However, Mendez et al demonstrated that delivery of IL-2 treated killer cells to burned mice, in combination with indomethacin, improved survival after cecal ligation and puncture.¹⁸

Restoration of host immunocompetence continues to be a primary goal in managing patients with significant thermal injury. Correcting defects in cell-mediated immunity has been attempted with such immunomodulators as cimetidine, ibuprofen, and cyclophosphamide,¹⁹ but most investigators believe that only expeditious removal

of the burn wound, with functional closure, can adequately restore host immunity. Although early burn wound excision was first proposed by Young in 1942,²⁰ acceptance of these principles was not immediate, due in part to limitations in resuscitation, anesthesia, perioperative monitoring, and grafting techniques. The development of effective topical antimicrobials in the 1960's greatly reduced the incidence of burn wound sepsis and decreased the need for early burn wound excision. Furthermore, many of the studies which did show improved outcome were retrospective, uncontrolled, and limited in population size. However, better designed clinical trials emerged in the 1980's to demonstrate a significant reduction in mortality for children,^{4,5} patients without inhalation injury,⁵ and physiologically stable patients with large full-thickness burns.⁷ Remaining controversies include the effect of excision on metabolic needs,²¹ the timing of excision, and the management of burns of indeterminate depth.⁶

Our finding that removal of the burn eschar improves CTL function supports the clinical practice early wound excision. In this experiment, mice received a 40% TBSA contact burn and underwent total, partial, or no excision of the wound three days postburn. CTLs harvested two weeks after injury were co-cultured with allogeneic stimulators and tested on ⁵¹Cr-labeled targets for allospecific cytotoxicity. Complete excision of the burn wound was associated not only with improved CTL function, but also decreased mortality. While partial wound excision also improved survival, CTL function remained suppressed, suggesting that wound excision provides additional benefits not necessarily related to improved CTL activity. The mechanism for improved CTL function following complete wound excision remains speculative but could involve altered circulation of CTLs away from the burn, improved alloantigen processing, or increased frequency of CTL precursors.

Other experiments support the immunologic benefits of early burn wound excision and coverage. Echinard et al demonstrated that early excision in guinea pigs blunted the catabolic response postburn and improved thymic DNA synthesis.²² Non-specific parameters of cell-mediated immunity, such as delayed-type hypersensitivity (DTH) and host-vs-graft reactions, are also improved following early excision.¹⁰ Tchervenkov et al examined the timing of wound excision in inbred rats and found that DTH and neutrophil delivery were restored to normal when animals underwent complete excision one day postburn; excision three days after injury partially restored DTH, while excision one week postburn had no effect.²³ More specific assays of cell-mediated immunity also support the concept of early excision. In a series of human subjects with a mean burn TBSA of 43%, Stratta et al reported that complete burn wound closure, performed early after patient resuscitation, was more effective than partial closure in reversing the suppressive effect of burn serum on lymphocyte immunocompetence in the mixed lymphocyte reaction.²⁴

Even though early, complete wound excision improved CTL function and survival in this experiment, the authors of this study are cautious to apply these principles directly to clinical practice. A considerable body of literature exists to question the functional, metabolic, and immunologic benefits of aggressive burn wound excision. In a murine model of thermal injury, Jacobson and Baker observed that very early excision (<24 hours postburn) exacerbated host immunosuppression and decreased survival.²⁵ Furthermore, excision during or shortly after resuscitation does not allow for an accurate determination of final wound depth and size, possibly resulting in excision of partial-thickness burns that would have benefited from more conservative treatment. Nevertheless, the results of this study support the concept of early burn wound excision and coverage. Improved CTL activity after early excision may help to restore host immunocompetence and may decrease the infectious complications of thermal injury.

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TABLE

Table. The effect of wound excision on mortality and weight change. Both total and partial excision contributed to significantly improved survival after 40% TBSA burn injury. Although total and partial excision mitigated postburn weight loss, these differences were not statistically significant.

| Experimental Groups | Number of Animals n=38 | Mortality (%) | Weight Change (%) 14 days postburn |
|---------------------|---------------------------|-----------------------------|---------------------------------------|
| unburned sham | 7 | 0% | 104% |
| total excision | 9 | 0% | 101% |
| partial excision | 10 | 10% | 99% |
| no excision | 12 | 42% | 95% |
| p value | | p<0.05 by χ -square | p=0.08 by ANOVA |

TABLE LEGEND

Table. The effect of wound excision on mortality and weight change. Both total and partial excision contributed to significantly improved survival after 40% TBSA burn injury. Although total and partial excision mitigated postburn weight loss, these differences were not statistically significant.

FIGURES

Figure 1. The effect of burn injury on CTL function, 4 days after in vitro stimulation.
 $*p < 0.05$ vs 0% burn, 40% burn; $**p < 0.01$ vs 0% burn, 20% burn.

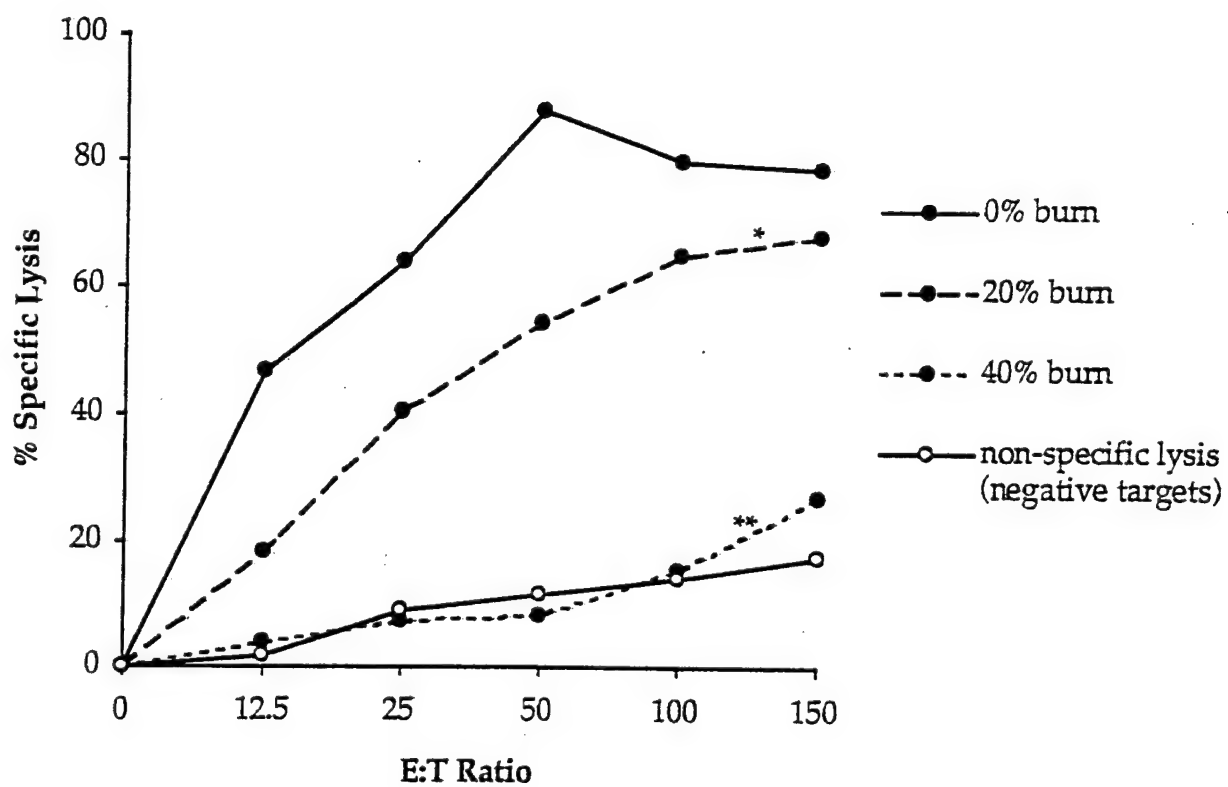


Figure 2. The effect of burn injury on CTL function, 5 days after in vitro stimulation.
 * $p < 0.01$ vs 0% burn, 40% burn; ** $p < 0.01$ vs 0% burn, 20% burn.

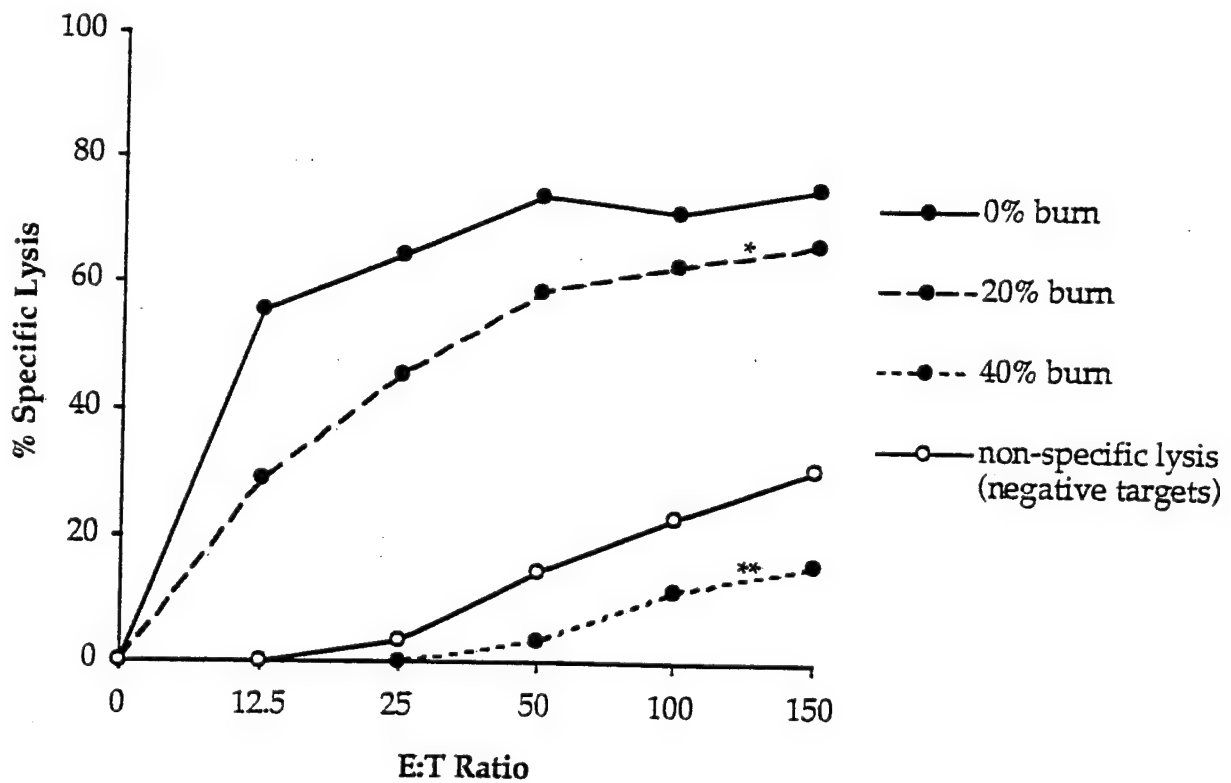


Figure 3. The effect of burn injury on CTL function, 6 days after in vitro stimulation.
 * $p < 0.05$ vs 0% burn, 40% burn; ** $p < 0.01$ vs 0% burn, 20% burn.

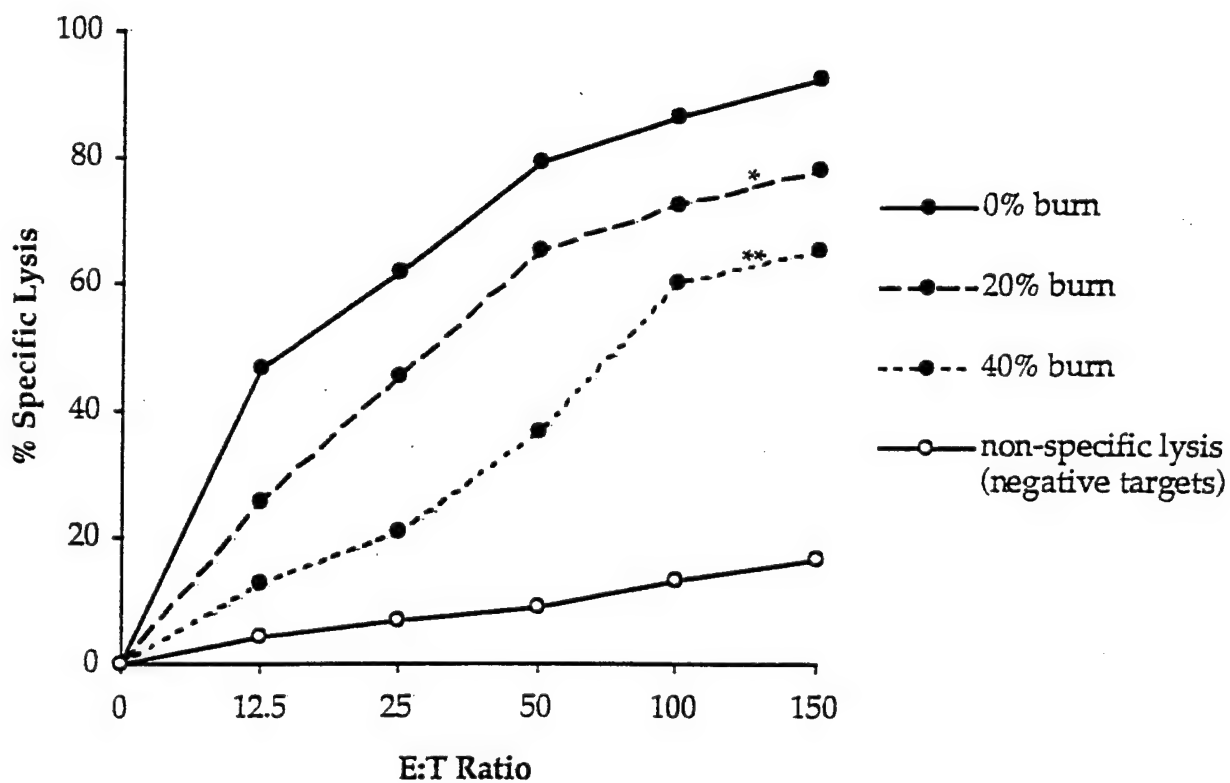


Figure 4. The effect of burn wound excision on CTL function, 3 days after in vitro stimulation. * $p < 0.05$ vs partial excision, no excision; ** $p < 0.05$ vs unburned sham, total excision.

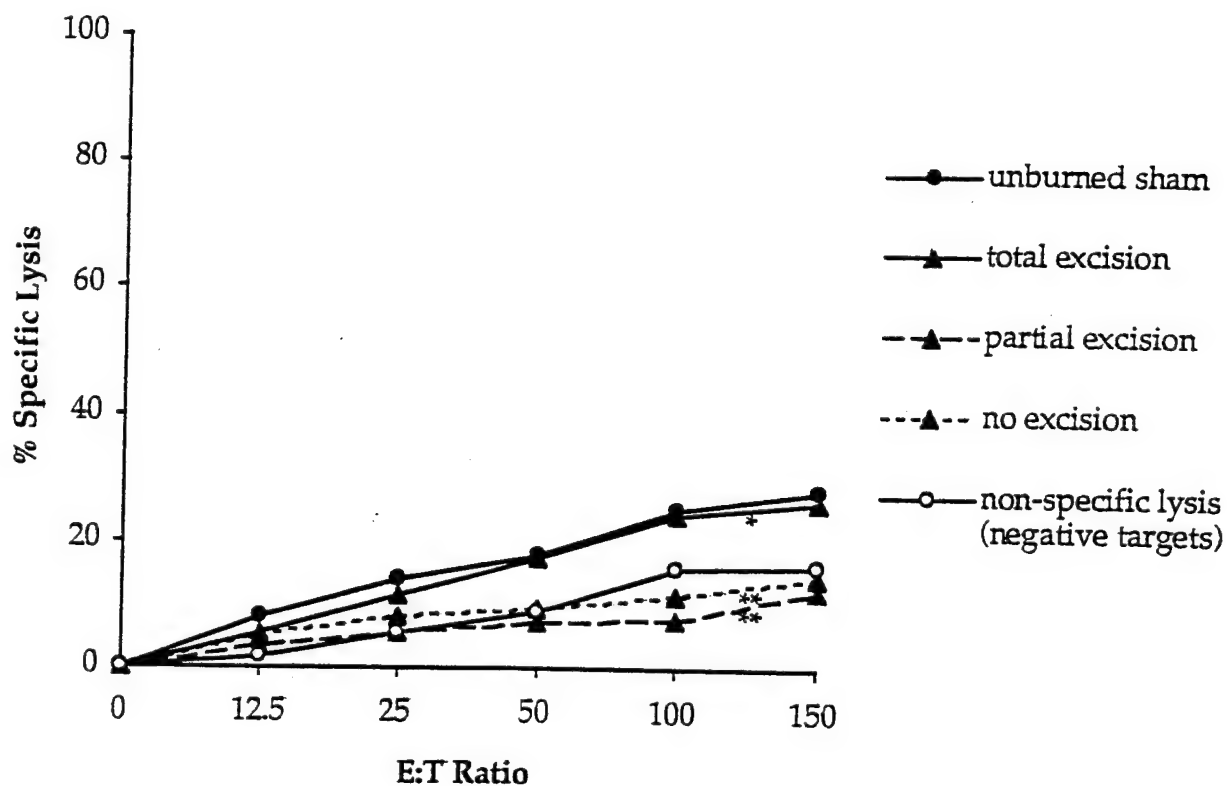


Figure 5. The effect of burn wound excision on CTL function, 4 days after in vitro stimulation. * $p < 0.01$ vs unburned sham, partial excision, no excision; ** $p < 0.01$ vs unburned sham, total excision.

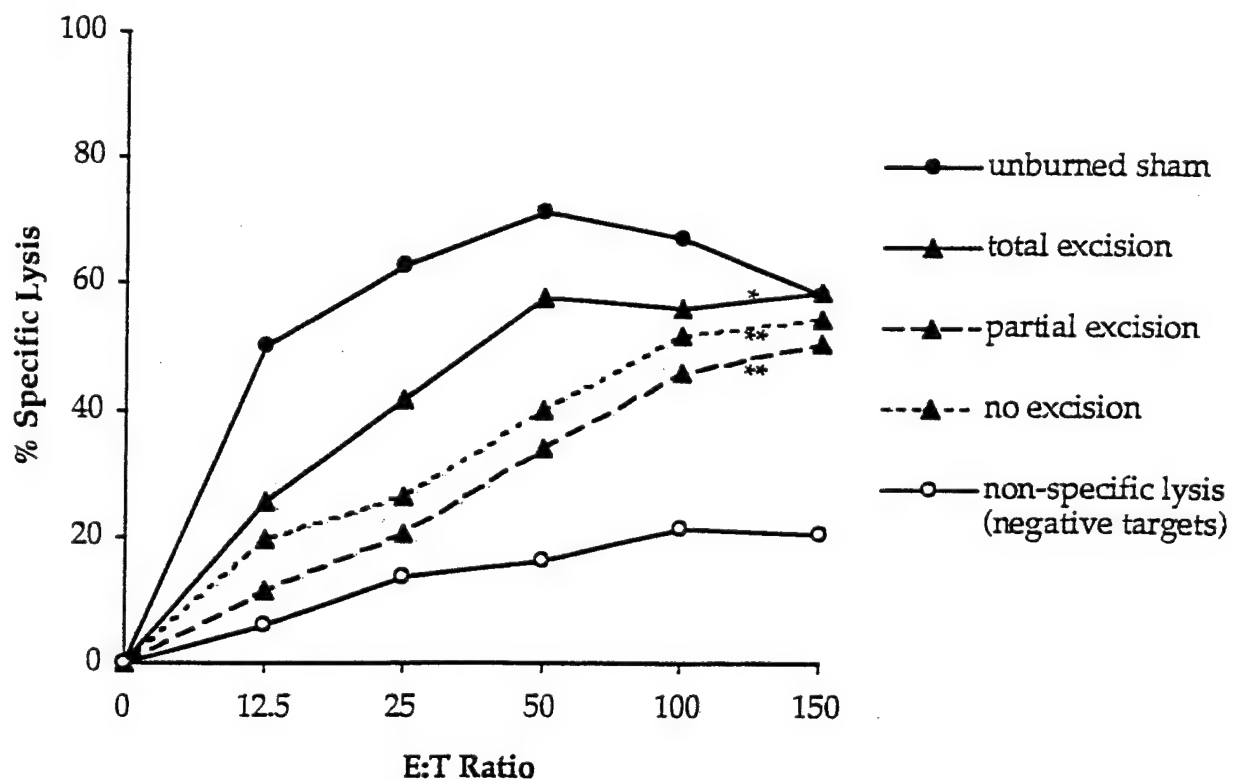


Figure 6. The effect of burn wound excision on CTL function, 5 days after in vitro stimulation. * $p < 0.01$ vs unburned sham, partial excision, no excision; ** $p < 0.01$ vs unburned sham, total excision.

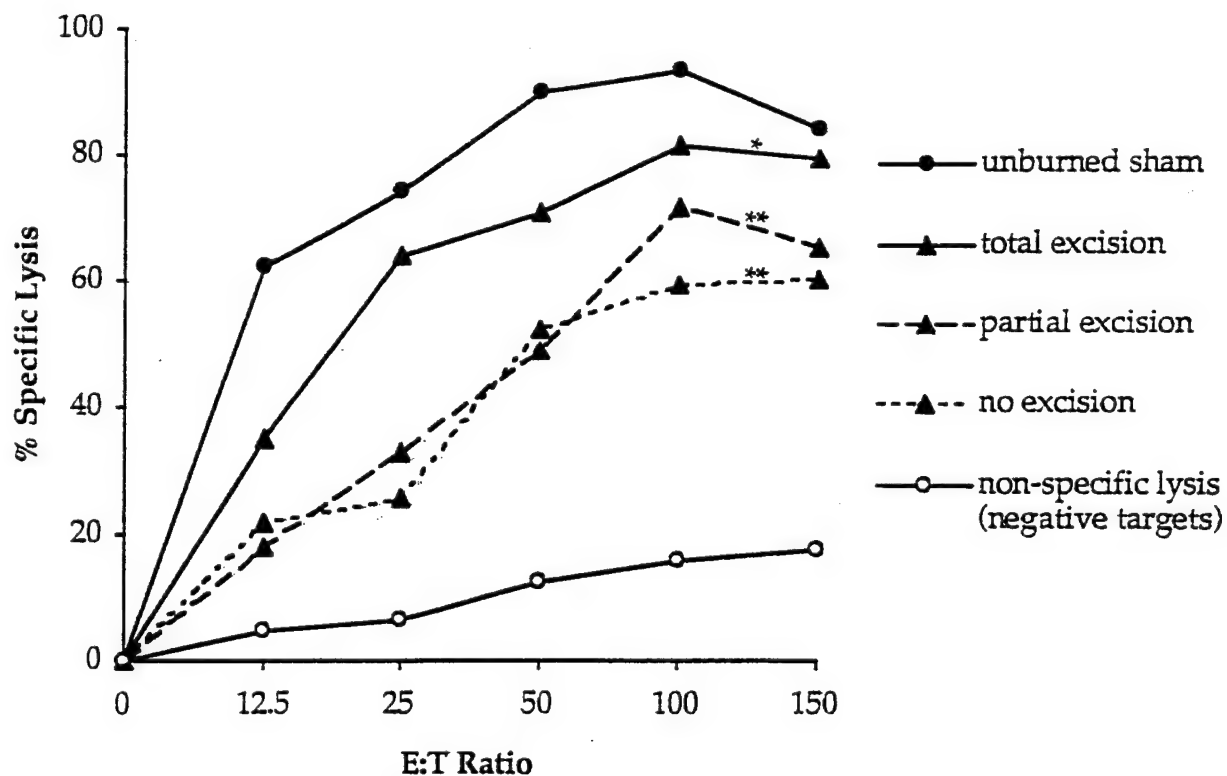


Figure 7. The effect of burn wound excision on CTL function, 6 days after in vitro stimulation. * $p < 0.01$ vs unburned sham.

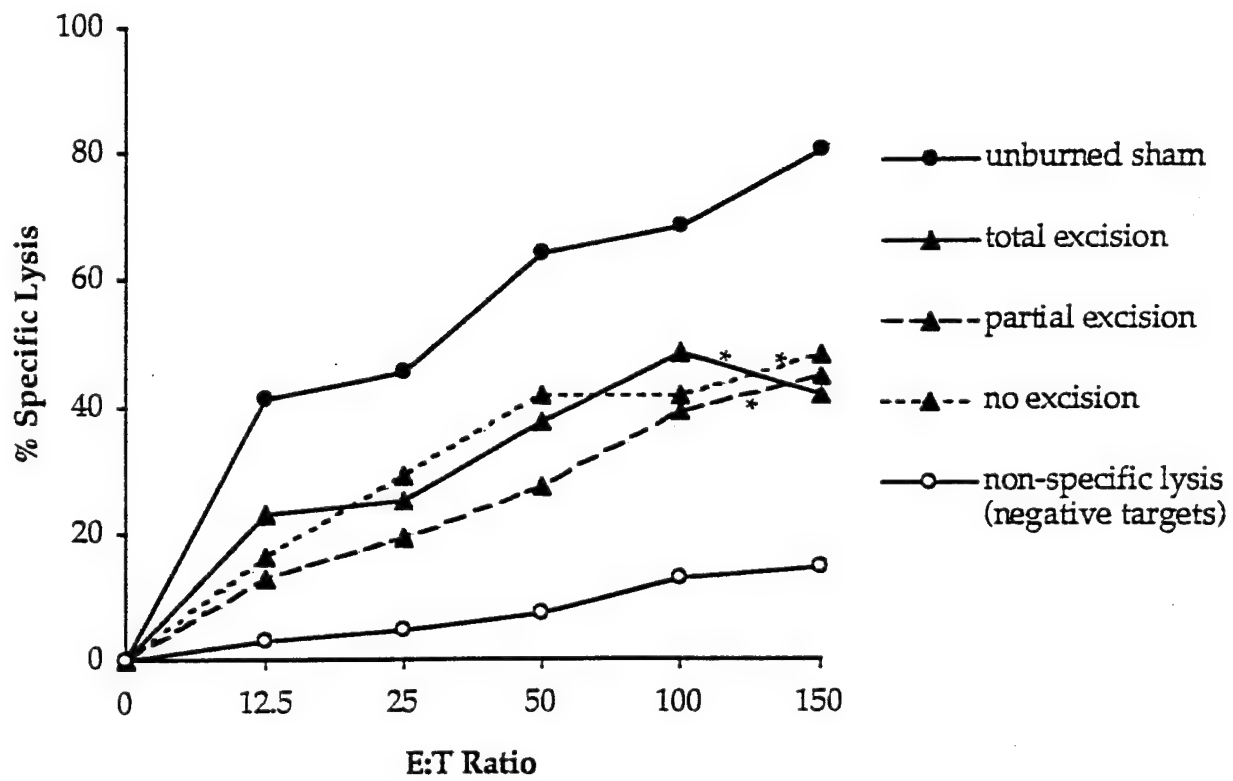


Figure 8. The effect of burn wound excision on CTL function, 7 days after in vitro stimulation. * $p < 0.01$ vs unburned sham, partial excision, no excision; ** $p < 0.05$ vs unburned sham, total excision, no excision; *** $p < 0.05$ vs unburned sham, total excision, partial excision.

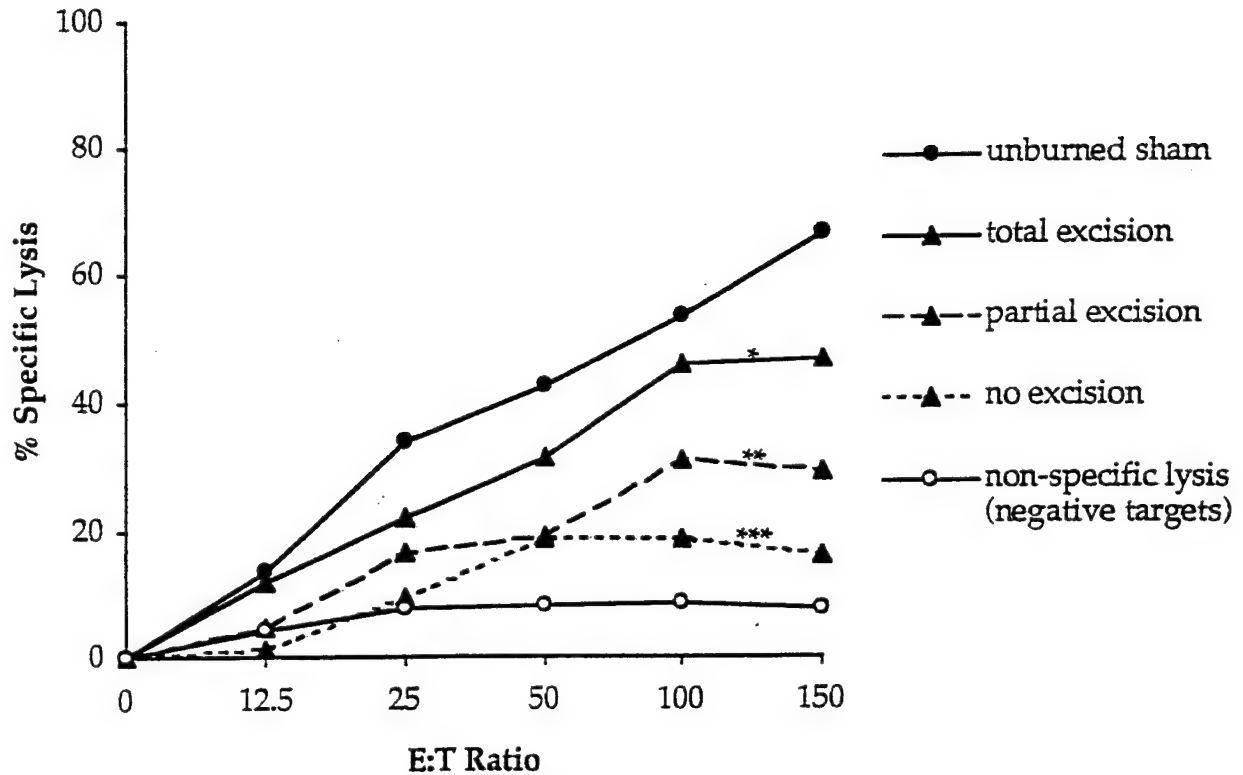


FIGURE LEGENDS

Figure 1. The effect of burn injury on CTL function, 4 days after in vitro stimulation. * $p < 0.05$ vs 0% burn, 40% burn; ** $p < 0.01$ vs 0% burn, 20% burn.

Figure 2. The effect of burn injury on CTL function, 5 days after in vitro stimulation. * $p < 0.01$ vs 0% burn, 40% burn; ** $p < 0.01$ vs 0% burn, 20% burn.

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Figure 7. The effect of burn wound excision on CTL function, 6 days after in vitro stimulation. * $p < 0.01$ vs unburned sham.

Figure 8. The effect of burn wound excision on CTL function, 7 days after in vitro stimulation. * $p < 0.01$ vs unburned sham, partial excision, no excision; ** $p < 0.05$ vs unburned sham, total excision, no excision; *** $p < 0.05$ vs unburned sham, total excision, partial excision.

The Relationship Between Interferon- γ and Keratinocyte Alloantigen Expression After Burn Injury

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Running title: Burn injury and cultured keratinocyte allografts

MINI-ABSTRACT

Cultured keratinocyte allografts have prolonged survival in patients with thermal injury, but it is unclear if this delayed rejection is due to defects in host responsiveness or decreased graft immunogenicity. We report that significant burn injury decreases graft levels of interferon- γ and inhibits the expression of Class II alloantigen. This provides a mechanism for limited allograft tolerance following thermal injury.

ABSTRACT

Background: Cultured keratinocyte (CK) and cadaveric skin allografts have prolonged survival in patients with massive thermal injury. It is unclear if this delayed rejection is due to impaired host responsiveness or decreased graft immunogenicity. Although burn injury has been shown to decrease parameters of allograft response, no studies have examined the effect of burn injury on alloantigen expression. This study investigated the effect of burn size on Class II antigen expression in CK allografts, as well as on tissue levels of interferon- γ (IFN- γ), the principle regulator of alloantigen expression. **Methods:** Anesthetized CBA mice (n=64) received a 0%, 20% partial-thickness (PT), 20% full-thickness (FT), or 40% FT contact burn. 48 hours later, wounds were partially excised and covered with CK allografts from C57BL/6 donors. Five days post-burn, grafts were analyzed for donor-specific Class II antigen. Protein expression was determined by Western immunoblotting and quantified with video densitometry. Wound, serum, and unburned skin levels of IFN- γ were determined by ELISA. Groups were compared by Fisher's ANOVA. **Results:** As burn size increased, Class II antigen expression decreased ($p<0.001$). This corresponded with decreased wound and skin levels of IFN- γ after 40% burn ($p<0.05$); however, wound IFN- γ was significantly elevated after 20% PT and FT burns ($p<0.01$). Serum IFN- γ increased as burn size increased ($p<0.01$). **Conclusions:** Burn injury decreases the antigenicity of CK allografts, which partly explains delayed allograft rejection after burn injury. Although wound IFN- γ increases after minor thermal injury, the profound decrease in wound and skin IFN- γ after a major burn corresponds with diminished Class II antigen expression. The decreased availability of IFN- γ after major thermal injury provides a mechanism for limited allograft tolerance.

INTRODUCTION

Cultured keratinocyte (CK) allografts have been proposed as a skin replacement in patients with massive thermal injury.¹⁻³ These patients represent a significant challenge to the burn surgeon because early excision and permanent closure of the burn wound may not be possible. The lack of donor sites limits the quality and quantity of autologous skin that can be harvested for wound coverage. Full-thickness allografts can be used as a temporary biologic dressing, but these grafts are eventually rejected unless the host remains immunosuppressed.⁴

CK allografts are particularly attractive due to their inherently limited immunogenicity. These epidermal sheets do not contain passenger leukocytes, as highly antigenic Langerhans cells found in skin do not persist in tissue culture.⁵ Furthermore, keratinocytes do not constitutively express major histocompatibility complex (MHC) Class II antigen,⁶ which is critically involved in allograft recognition and rejection. Additionally, CK allografts fail to elicit cytotoxic antibody once grafted and do not generate a mixed-lymphocyte response.⁷

Recent work, though, suggests that CK allografts are more immunogenic than previously believed. CK allografts prime the unburned host for accelerated second-set rejection and activate cytotoxic T lymphocytes.⁸ Additionally, keratinocytes will express MHC Class II antigen when exposed to interferon-gamma (IFN- γ) in vitro⁹ or in vivo.¹⁰ Repeated application of these grafts in the immunocompetent host may create a chronic inflammatory state counterproductive to wound healing. Although CK allografts are not acutely rejected, the long-term survival of these grafts is unknown, as they are gradually replaced by host keratinocytes.¹¹

The immunosuppression of thermal injury, however, may decrease the immunogenicity of CK allografts and improve their function as a permanent skin replacement in burn wound coverage. We have recently demonstrated that burn injury selectively impairs host sensitization to CK allografts, compared to full-thickness allografts.^{12,13} While this decreased host responsiveness may be due to defects in cell-mediated immunity, perhaps burn injury, with its altered cytokine profiles, affects the antigenicity of the actual grafts. The purpose of this study is to determine if burn injury inhibits the induction and expression of MHC Class II antigens in CK allografts. Furthermore, we will investigate the effect of burn injury on tissue levels of IFN- γ , the principle cytokine which regulates expression of MHC Class II antigen.

MATERIALS AND METHODS

Experimental design. This study examined the effect of burn injury on MHC Class II antigen expression in CK allografts, as well as the effect of burn injury on the production of IFN- γ . In the first experiment, CBA mice (n=36) received one of four burn wounds: 0% total body surface area (TBSA), 20% partial-thickness (PT), 20% full-thickness (FT), or 40% FT. Forty-eight hours post-burn, wounds were partially excised and covered with CK allografts, which were biopsied three days later. Specimens were then analyzed for donor-specific MHC Class II antigen by Western immunoblotting.

In the second experiment, CBA mice (n=28) received 0%, 20% PT, 20% FT, and 40% FT burn injuries, which again were partially excised and covered with CK allografts derived from C57BL/6 mice. Three days after grafting (five days after burn injury), serum, wound, and skin samples were collected and processed to determine

levels of IFN- γ . An enzyme-linked immunosorbent assay (ELISA) was used to measure levels of this cytokine.

Animal protocols. Fifteen to twenty gram, four-week-old female CBA/J (H-2^k) mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) were used as graft recipients in both the MHC Class II antigen and IFN- γ experiments. Graft donors were age/weight-matched female C57BL/6 (H-2^b) mice (Charles Rivers Laboratories, Wilmington, MA). All protocols were approved by the UNC Committee on Animal Research and were in accordance with NIH guidelines.

Burn injury. Animals were anesthetized with methoxyflurane (Pitman-Moore, Washington Crossing, NJ) and circumferentially clipped. Burn injury was accomplished by the application of a 65g copper rod, previously heated to 100°C, to the animal's back and flank for 10 seconds in FT wounds and 5 seconds in PT wounds. Each application represented 10% of the animal's total body surface area, with four applications necessary to produce a 40% burn. The contact burn described in this model creates an injury of predictable depth and border, permitting precise wound excision and grafting.

All mice were then resuscitated with intraperitoneal lactated Ringer's (0.1ml/g body weight) and were given subcutaneous morphine sulfate (3 μ g/g body weight) for post-burn pain control. Animals were returned to individual cages to feed ad libitum. Mice receiving the 0% sham burn underwent all of these interventions, with the exception of the application of the copper rod.

Keratinocyte cultures. Approximately three weeks prior to burn injury, keratinocyte cultures were prepared using methods modified from the technique

described by Rheinwald and Green.¹⁴ All tissue culture media and additives were obtained from the Lineberger Cancer Center (Chapel Hill, NC), except where noted.

Tail skins were obtained from C57BL/6 donors, washed with 70% ethanol, and stored overnight in Dulbecco's modified MEM (DMEM) supplemented with 0.1% penicillin/streptomycin. The skin was then incubated in 0.25% trypsin (Sigma Chemical Co., St. Louis, MO) for two hours at 37°C and in 5% CO₂. The epidermis was separated from the dermis and vortexed to create a single cell suspension. These keratinocytes were co-cultured at a 2:1 ratio with a growth-arrested feeder layer of murine connective tissue cells (LTK, H-2^k), previously exposed to mitomycin C (Sigma Chemical Co., St. Louis, MO) for 45 minutes. Cells were grown in a solution of DMEM and Ham's F-12, which contained 5% FBS (Hyclone Laboratories Inc., Logan, UT), cholera enterotoxin (Schwartz Mann/ICN Biochemical Inc., Costa Mesa, CA), hydrocortisone 0.4mg/ml, transferrin 5.0mg/ml, and insulin 5.0mg/ml (all from Sigma Chemical Co., St. Louis, MO), and amphotericin 5.0mg/ml (E. R. Squibb and Sons Inc., Princeton, NJ). Media was changed every 2-3 days, and epidermal growth factor 10.0 ng/ml (Collaborative Research Inc., Bedford, MA) was added after the first media change. Seven to ten days after plating, LTK cells were removed by differential trypsinization and keratinocytes were grown to confluence.

Grafting Procedure. Forty-eight hours after burn injury, wounds were partially excised and grafted with CK allografts. This time period was selected to approximate the clinical course of early excision and grafting in humans. Furthermore, most of the burn wound was left intact to ensure continued host immunosuppression. Burn wound depth was confirmed by histologic study of biopsies obtained at the time of excision.

Upon reaching confluence, CK allografts were released from culture dishes with the enzyme dispase (Boehringer Mannheim, Germany), placed on petroleum gauze "basal side up," and stored in the incubator until grafting. C57BL/6 allografts were then grafted onto the left flank of CBA recipients, after excision of the wound to musculoskeletal fascia. Gauze-backed grafts were tucked under the surrounding wound edge "basal side down" and covered with a Vigilon hydrophilic dressing (C. R. Bond Inc., Berkeley Heights, NJ), which was secured with a stretch fabric bandage and skin staples.

Wound assessment. On post-burn day five, three days after grafting, wounds were inspected, photographed, and biopsied with a 3mm punch probe. Structural persistence of CK allografts was determined by fixing the tissue in 10% formalin; biopsies were later dehydrated, infiltrated with methyl-methacrylate, embedded in a gelatin capsule, sectioned on a GB4A microtome, and stained with methylene-blue acid-fuchsin for histochemical analysis.

Western immunoblotting. Donor-specific MHC Class II antigen expression in CK allografts was determined by Western immunoblotting. Wounds were biopsied three days after grafting, to correspond with maximal *in vivo* Class II expression that we have previously observed and reported.⁸ Protein lysates were obtained by solubilizing biopsies in 200 μ l of 0.001 M Tris-HCl, pH 7.4, 0.150 M NaCl, 0.5% NP-40, 1mM PMSF. Specimens were then vortexed, incubated on ice for 15 minutes, centrifuged for 15 minutes at 4°C, stored at -80°C, and standardized for protein concentration prior to immunoblotting. Polyacrylamide 12% SDS gels were created with a Bio-Rad Model 360 vertical slab mini-cell and a Model 361 casting chamber. Detergent-extracted specimens and molecular-weight markers were run on one-

dimensional gels and transferred to nitrocellulose, which was then blocked with 1% BSA. The blots were incubated with KL295, a murine monoclonal antibody that recognizes the denatured 30 kD α chain of H-2^b (but not H-2^k) MHC Class II antigen. Following primary antibody exposure, blots were incubated with goat anti-mouse antibody labeled with alkaline phosphatase, to mark any murine immunoglobulin present on the Western blot.

Video densitometry. Expression of donor MHC Class II antigen was quantified with video densitometry. Western immunoblots were scanned with Macintosh video recording equipment (Apple Computer, Cupertino, CA), stored as video images in Power Point software (Microsoft, Redmond, WA), and analyzed with Gel Capture software (NIH, Bethesda, MD). The amount of MHC Class II antigen present was objectively determined by measuring the pixel density for each protein band. In an effort to minimize gel-to-gel variability, we compared antigen expression by calculating band strength as a proportion of the protein standard [(absolute pixel density of protein band)/(background density of the protein marker lane)].

Collection of tissue samples for IFN- γ levels. Three days after grafting (five days after burn injury), wounds were analyzed for IFN- γ by mincing a 10mm punch biopsy of the CK allograft in phosphate buffered saline. Biopsies were then sonicated, vortexed, and centrifuged at 1500 RPM for five minutes. Supernatants were collected and frozen at -80°C until the IFN- γ assay was performed. Unburned skin from the right ear was similarly processed. Serum IFN- γ was determined by collecting blood via retro-orbital puncture, allowing the clot to separate, centrifuging the serum, and freezing the supernatant at -80°C.

Determination of IFN- γ levels. IFN- γ levels in CK allografts, unburned skin, and serum were measured with a commercially available ELISA kit (Endogen, Inc., Boston, MA), sensitive and specific for murine IFN- γ between concentrations of 15-3000 pg/ml. Samples were tested in duplicate and compared to known IFN- γ standards.

Statistical Analysis. Mean MHC Class II antigen expression was compared between burn groups by ANOVA and Student's T test. Mean IFN- γ levels in grafted wounds, serum, and unburned skin were compared between burn groups by Fisher's Protected LSD. Error bars in Figures 3-6 represent standard error of the mean, and each burn group contains a sample size of approximately seven surviving animals.

RESULTS

Animal survival. The graduated severity of our burn model can be appreciated by observing the mortality of each group. In these and other experiments, survival rates for each of the burn groups were as follows: 0% burn, 100% survival; 20% PT burn, 95% survival; 20% FT burn, 90% survival; 40% FT burn, 65% survival. In this study, autopsies of the animals who did not survive after post-burn day five showed mesenteric edema and ischemia; no wound infections were grossly apparent.

Histochemical studies. All of the wounds had either macroscopic or microscopic evidence of CK allograft persistence three days after grafting. Acid-fuchsin histology revealed the presence of acellular keratin sheets, stratification of keratinocytes with a basal layer, and good apposition of the CK allografts to the chest wall, as illustrated in Figure 1. Burned animals tended to have a more pronounced mononuclear cell infiltrate between the CK allografts and muscle fascia, consistent with the increased inflammation expected of burn injury.

MHC Class II alloantigen expression. To assess the induction and expression of MHC Class II antigen *in vivo*, we performed Western immunoblots on allograft biopsies from mice whose burn wounds had been partially excised and covered with allogeneic cultured keratinocytes. We observed that burn injury decreased MHC Class II antigen expression as a function of burn size. The immunoblot in Figure 2 demonstrates donor-specific Class II protein expression from all four burn groups. Direct comparison with the accompanying video densitometrogram enables the viewer to quantify the intensity of each band, as measured by pixel density. In this experiment, positive controls included spleens and full-thickness skin from C57BL/6 mice, while negative controls included spleens and full-thickness skin from CBA mice, *in vivo* CK autografts, and *in vitro* C57BL/6 keratinocyte sheets.

As depicted in Figure 3, MHC Class II alloantigen expression decreased as a function of burn depth and burn size. Mean antigen expression for each burn condition was determined by scanning protein bands with video densitometry, measuring absolute pixel density for each band, adjusting for gel-to-gel variability by dividing pixel density by background density, and averaging these adjusted densities for each group. Mean antigen expression for each burn condition was as follows: 0% sham, 2.81; 20% PT burn, 2.49; 20% FT burn, 1.54; 40% FT burn, 0.47. Both 20% FT and a 40% FT burn injury significantly decreased expression of MHC Class II alloantigen, compared to the sham controls ($p < 0.001$). A 20% PT burn also inhibited Class II expression but this difference was not statistically significant. This relationship between burn injury and alloantigen expression corresponds with the immunoblot from Figure 2, in which biopsies from all four groups were developed on same gel.

Tissue levels of IFN- γ . With evidence that burn injury decreases antigen expression in CK allografts, we decided to study the mechanism responsible for the induction and regulation of MHC Class II antigen. IFN- γ , which has been implicated as a critical mediator of Class II antigen expression, was measured after thermal injury in allografted wounds, serum, and unburned skin. Both 20% PT and 20% FT burns significantly increased wound levels of IFN- γ , while a 40% injury significantly decreased levels of this cytokine, compared to the unburned, grafted controls (Figure 4). Mean wound levels of IFN- γ (in pg/ml) were as follows: 0% sham, 167.4; 20% PT burn, 409.3; 20% FT burn, 322.6; 40% FT burn, 47.7. Although a minor burn injury increased levels of wound IFN- γ , burn injury inhibited wound IFN- γ as the severity of injury increased, corresponding to impaired alloantigen expression.

In this model of burn injury, serum levels of IFN- γ increased as the size of the burn injury increased. Mean serum IFN- γ levels for 0%, 20% PT, 20% FT, and 40% FT burns were 17.9, 36.1, 34.8, and 117.0, respectively. While both 20% PT and 20% FT burns modestly increased serum IFN- γ , 40% FT burn injury increased levels of this cytokine six-fold (Figure 5). Despite the fact that serum levels of IFN- γ increased as a function of burn size, we observed that IFN- γ levels in unburned skin decreased as burn size increased. Mean skin levels of IFN- γ for 0%, 20% PT, 20% FT, and 40% FT burns were 80.4, 64.1, 68.5, and 15.2, respectively. Both 20% PT and 20% FT burn injuries slightly decreased levels of skin IFN- γ , and a 40% FT injury significantly decreased levels of this cytokine (Figure 6).

In summary, major thermal injury increases systemic, circulating levels of IFN- γ but decreases delivery and/or local production of this cytokine. Although wound IFN- γ increases after minor burn injury, the profound decrease in wound and skin IFN- γ after

major burn injury corresponds with diminished Class II alloantigen expression. The relationship between burn injury, alloantigen expression, and IFN- γ levels can be observed in Table 1, which provides a summary of these data.

DISCUSSION

In this study, we provide evidence that burn injury decreases the immunogenicity of CK allografts. Although keratinocytes do not normally express MHC Class II antigen, exposure to IFN- γ induces de novo expression of Class II antigen and imparts on keratinocytes the ability to initiate an alloimmune response.¹⁵ Burn injury, however, appears to inhibit induction of MHC Class II antigen in a burn-size dependent manner. Furthermore, decreased expression of MHC Class II antigen in large burns corresponds with diminished wound and skin levels of IFN- γ . The implication of these findings is that the immunosuppression of burn injury decreases the antigenicity of CK allografts, which might extend the long-term survival of these grafts and improve their potential as a permanent skin replacement.

The early excision and closure of massive burn wounds increases patient survival and improves functional outcome¹⁶ but remains a significant challenge for burn care providers. In 1983, Hefton and Shires reported the use of CK allografts in three patients who underwent tangential excision of deep partial-thickness injuries.¹ Early enthusiasm for these grafts was based on the principles that CK allografts could be grown and stored prior to injury,¹⁷ that the grafts function as a bioactive occlusive dressing,¹⁸ and that CK allografts have limited immunogenicity.¹⁹

CK allografts have also been promoted in the treatment of non-burn conditions, such as epidermolysis bullosa²⁰ and venous stasis ulcers,²² with the rationale that these

grafts secrete numerous growth factors that guide keratinocyte migration, increase angiogenesis, and participate in the formation of the extracellular matrix.²² Two recently published, controlled, blinded trials suggest that CK allografts may be useful in management of skin graft donor sites, by reducing pain and accelerating reepithelialization. Although allogeneic keratinocytes did not survive permanently, CK allografts decreased wound healing from 14 to 8 days,²³ reduced the interval required between repeated donor site harvests, and improved the quality of the reharvested skin.²⁴

However, numerous economical and technical limitations have prevented the widespread acceptance of CK allografts as a biologic material to be used in burn wound coverage. Cultured epidermal sheets cost approximately \$13,000 per 1% body surface area covered and require multiple applications because of unpredictable graft take.²⁵ CK allografts may develop a "neo-dermis" after several months,²⁶ but the lack of a dermal element certainly increases the mechanical fragility of recently grafted keratinocytes. Furthermore, allogeneic keratinocytes, when placed on partial thickness wounds, are gradually replaced by host keratinocytes, as determined by DNA fingerprinting,²⁷ and may be effective only when the wound can be repopulated by keratinocytes from deeper dermal elements. The fate of CK allografts when used to cover fascia or granulation tissue remains unknown.

Although CK allografts do not contain passenger leukocytes, these grafts are more immunogenic than originally believed. Keratinocytes express MHC Class II antigen in various autoimmune diseases,²⁸ after exposure to IFN- γ in vitro,⁹ and when grafted as epidermal sheets.⁸ Using a model of second-set rejection, originally described by Medawar in 1944,²⁹ we have demonstrated that CK allografts and full-thickness

allografts sensitize the unburned host with equal efficacy.⁸ Prior exposure to either graft decreases second-set allograft survival from 13 to 9 days. This priming occurs via activation of cytotoxic T lymphocytes and has possible clinical implications in patients who need extensive, repeated coverage with CK allografts.

Because burn injury produces numerous, specific defects in cell-mediated immunity, such as suppression of T lymphocyte activation,^{30,31} we suspected that burn injury might undermine the process of sensitization and ameliorate the immunogenicity of allogeneic keratinocytes. We recently reported that burn injury selectively interferes with priming by CK allografts, compared to full-thickness allografts.^{12,13} Second-set rejection in burned mice who had received CK allografts was significantly delayed. This current study examines the effect of burn injury on the antigenicity of CK allografts, and we propose that impaired priming by allogeneic keratinocytes may be due to down-regulation of MHC Class II antigen, which is critical in initiating the rejection response.

A possible mechanism for diminished alloantigen expression may be related to altered cytokine levels following burn injury. IFN- γ , which is a 20-25 kD polypeptide produced by activated T lymphocytes, normally upregulates MHC expression, making allografts, virally-infected cells, and tumors more susceptible to CTL-mediated lysis.³² Keratinocytes are not privileged from this process and can serve as both stimulators and targets of the immune response.³³ Although they lose T-cell activating ability in culture,³⁴ keratinocytes develop MHC Class II antigens and express increased Class I antigens when stimulated with IFN- γ .¹⁵ However, IL-1, TNF- α , and PGE₂, which are elevated after thermal injury,^{35,36} have been shown to either down-regulate the expression of Class II antigen or antagonize the effects of IFN- γ .³⁷⁻³⁹

The effect of burn injury on IFN- γ production, though, has yet to be fully defined. Our observation that burn injury decreases tissue levels of IFN- γ is consistent with previous research that implicates decreased IFN- γ as a cause of post-burn immunosuppression. In a series of classic experiments, Suzuki and Pollard demonstrated a biphasic depression in T cell production of IFN- γ 3-5 days and 3-5 weeks after burn injury.^{40,41} Furthermore, the size of the burn wound inversely correlated with the ability of T cells to generate IFN- γ in vitro.

Evidence for the importance of this cytokine in restoring immunocompetence after thermal injury and trauma can be noted in the ability of IFN- γ to decrease bacterial translocation in mice,⁴² improve natural killer cell function in rats,⁴³ and decrease infection-related deaths in humans.⁴⁴ Several investigators have suggested that susceptibility to infection after trauma may be related to impaired antigen detection via down-regulation of host MHC Class II antigen.^{45,46} Using flow cytometry to quantify HLA-DR expression, these authors have shown that depressed in vitro production of IFN- γ correlates with reduced monocyte Class II expression, which was noted as early as 24 hours after injury and lasted for two weeks. This report is consistent with our observation that a 40% burn injury inhibits Class II alloantigen expression, as well as allograft levels of IFN- γ .

Elevated graft levels of IFN- γ after a minor, partial-thickness injury may be due to local, pro-inflammatory effects of the burn wound. Our finding that even minor burn injury inhibits Class II expression, despite increased levels of IFN- γ , suggests that other important cytokines are involved in the regulation of Class II alloantigen. Although speculative, one explanation is that TNF- α and PGE₂, both of which are

increased post-burn, may antagonize the effects of IFN- γ and down-regulate Class II antigen expression.

Many studies have examined the effect of burn injury on serum cytokine levels, but very few have focused on systemic, circulating levels of IFN- γ . One group has recently reported that in burn patients, infection rates and injury severity positively correlated with plasma levels of both IFN- γ and IL-6.⁴⁷ This data, combined with our observation that burn injury increases serum levels of IFN- γ but decreases skin levels, implies that this immunoregulatory cytokine is not available for local utilization, despite elevated plasma levels. Major burn injury, through undescribed mechanisms, may interfere with the local production of IFN- γ or may interfere with tissue delivery. The increased serum IFN- γ levels observed after burn injury may also reflect compensatory production of this cytokine by circulating lymphocytes in an effort to restore immunocompetence.

In conclusion, we report that a significant burn injury, with its associated immunosuppression, decreases the immunogenicity of CK allografts by inhibiting expression of MHC Class II alloantigen. Furthermore, decreased expression of alloantigen corresponds with decreased levels of IFN- γ in grafted wounds. The decreased immunogenicity of CK allografts may minimize sensitization and allow for repeated applications in burn patients without alloantigen priming. Additionally, this decreased immunogenicity may improve the long-term survival of allogeneic keratinocytes and enable early, complete excision of full-thickness burn wounds. Understanding and further manipulating the mechanism of alloantigen induction may someday permit the use of CK allografts as a definitive, permanent, biologic skin replacement.

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FIGURE LEGENDS

Figure 1. Photomicrograph (100x magnification) of a CK allograft, five days after burn injury and three days after wound excision and grafting. This CK allograft is closely adherent to musculoskeletal fascia (under which unstained muscle fibrils are visible) and contains a stratified keratinocyte layer with a basal component. Also apparent is a newly formed keratin sheet, not observed in ungrafted keratinocyte cultures.

Figure 2. A. Western immunoblot depicting MHC Class II alloantigen expression following burn injury and grafting. Explanation of lanes, with abbreviations, is as follows: prot std (low molecular weight protein standards), + control (C57BL/6 splenocytes), - controls (CBA splenocytes and CBA skin), 0% (sham burn), 20% PT (20% total body surface area partial thickness burn), 20% FT (20% total body surface area full thickness burn), and 40% FT (40% total body surface area full thickness burn).

B. Video densitometrogram relating absolute pixel density of individual protein bands. Burn injury decreased alloantigen expression as burn size increased.

Figure 3. The effect of burn size on MHC Class II antigen expression in CK allografts, three days after excision and grafting. Each group contains approximately seven surviving animals. Immunoblots were scanned with video densitometry to quantify alloantigen expression, which is depicted graphically as mean adjusted pixel density. Error bars represent standard error of the mean. Both 20% FT and 40% FT burn injuries significantly inhibited Class II alloantigen expression. * $p < 0.05$ vs 0%; ** $p < 0.001$ vs 0%.

Figure 4. The effect of burn size on IFN- γ levels in grafted wounds. Both 20% PT and 20% FT injuries significantly increased wound IFN- γ , whereas 40% FT burn decreased wound IFN- γ . * $p < 0.05$ vs 0%, 20% PT, 20% FT; ** $p < 0.01$ vs 0%, 40% FT.

Figure 5. The effect of burn size on serum levels of IFN- γ . Although 20% PT and FT burns modestly increased serum IFN- γ , only 40% burn injury significantly increased serum levels of this cytokine. * $p < 0.01$ vs 0%, 20% PT, 20% FT.

Figure 6. The effect of burn size on IFN- γ levels in unburned skin. Both 20% PT and 20% FT burns slightly decreased local levels of IFN- γ , but only 40% burn injury significantly inhibited levels of this cytokine in unburned skin. * $p < 0.01$ vs 0%, 20% PT, 20% FT.

Figure 1. (photomicrograph)

Figure 2. (Western immunoblot with video densitometry)

Figure 3.

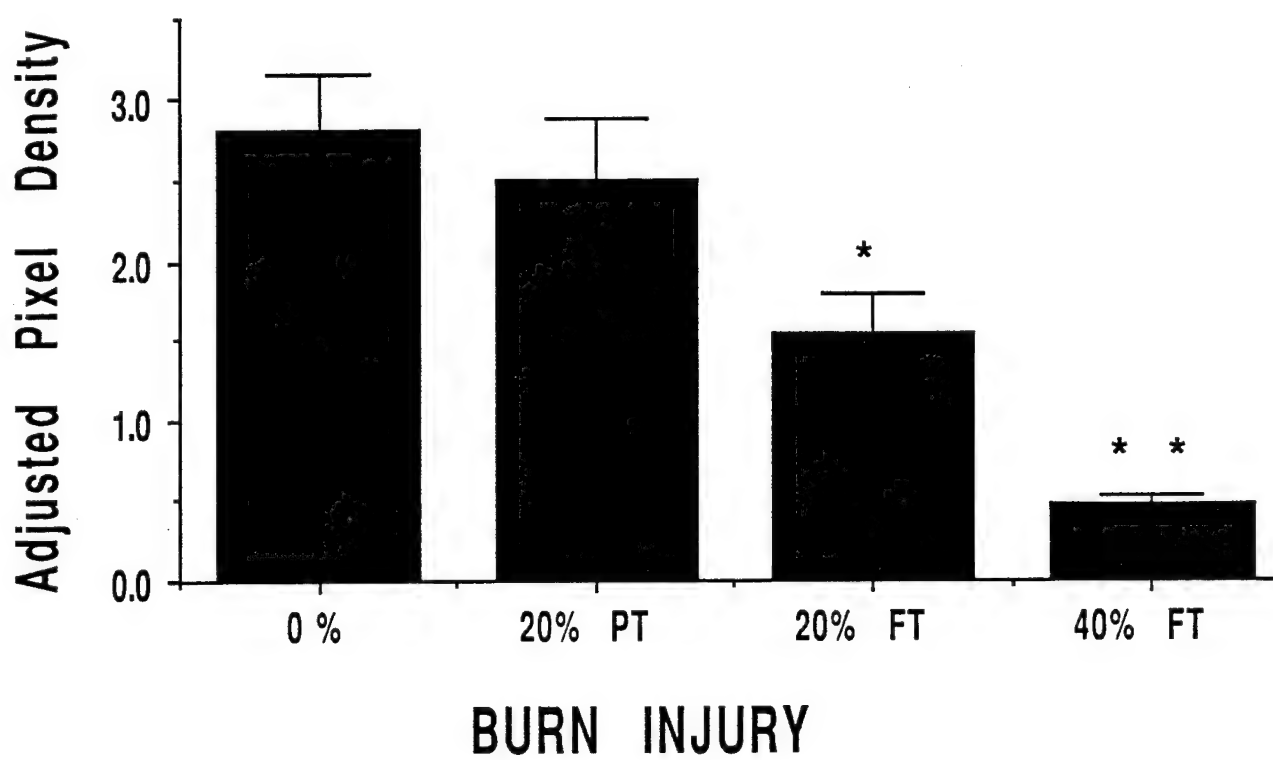


Figure 4.

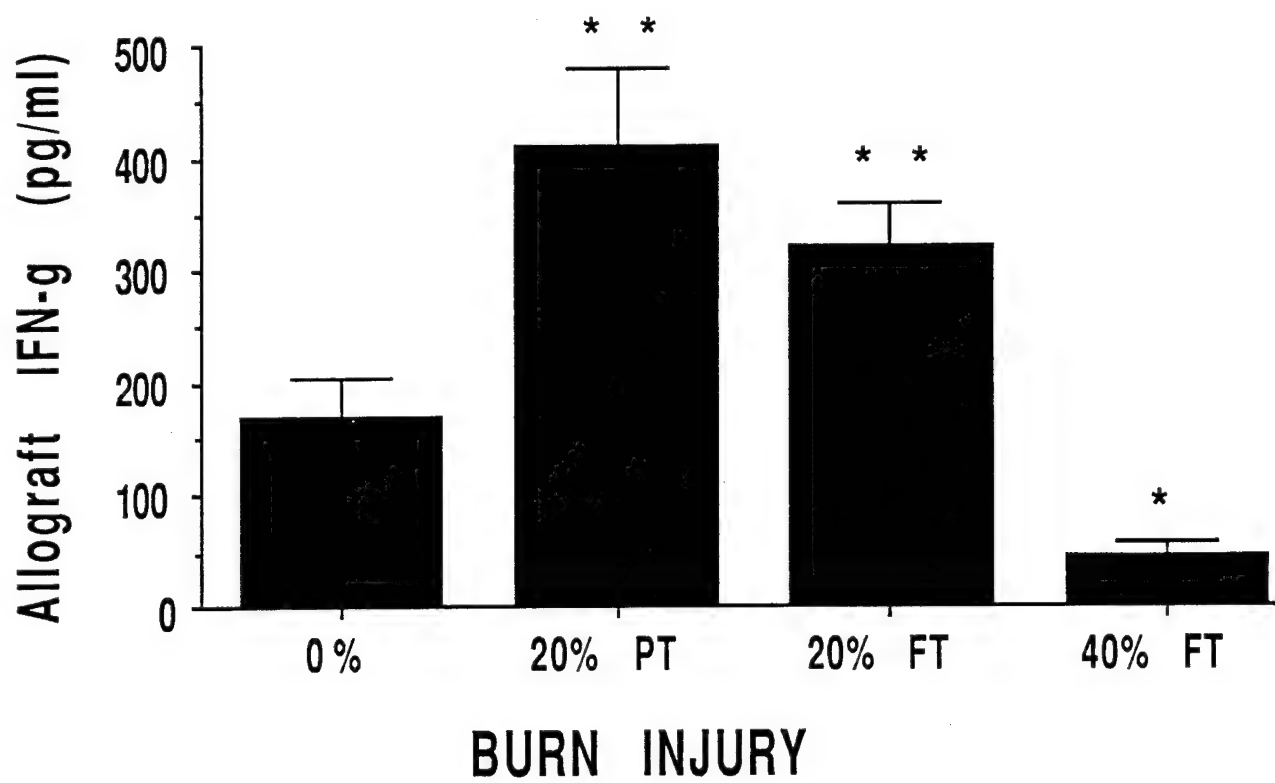


Figure 5.

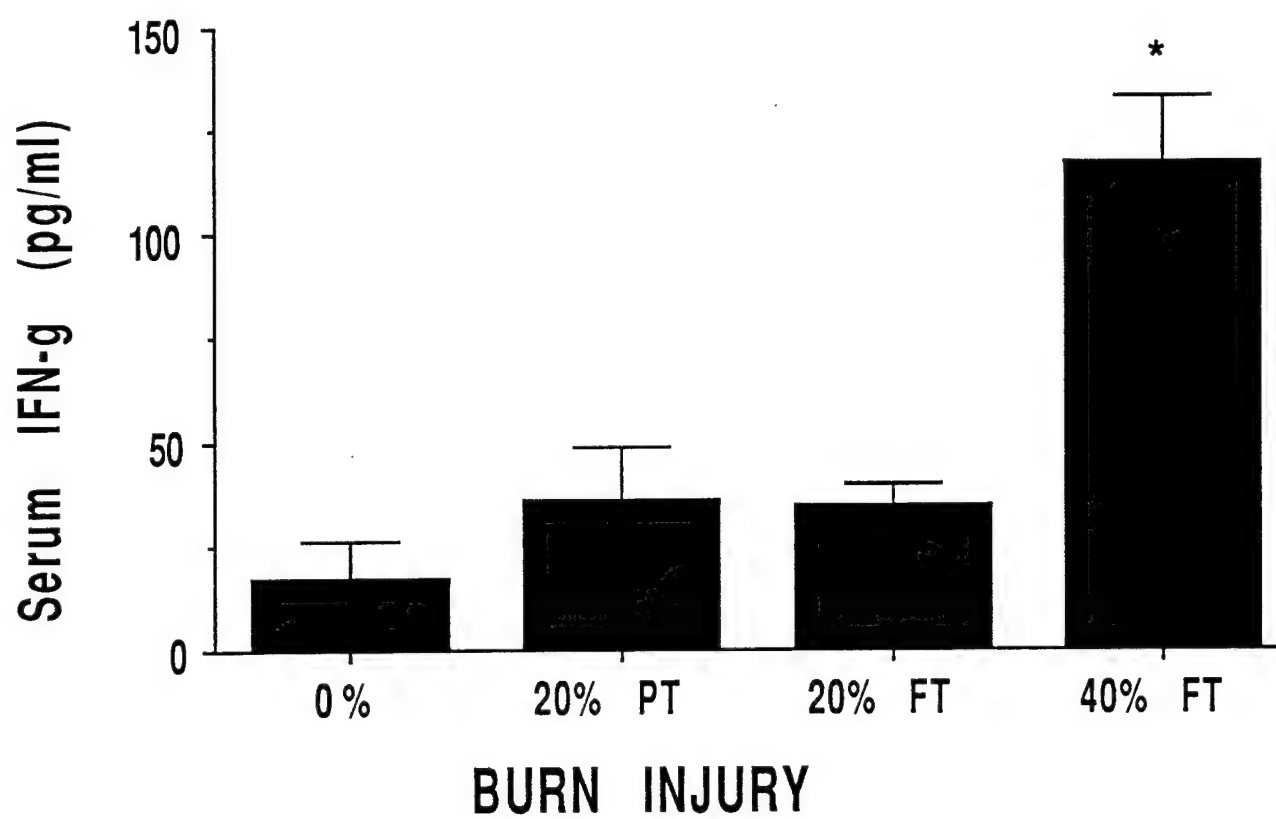


Figure 6.

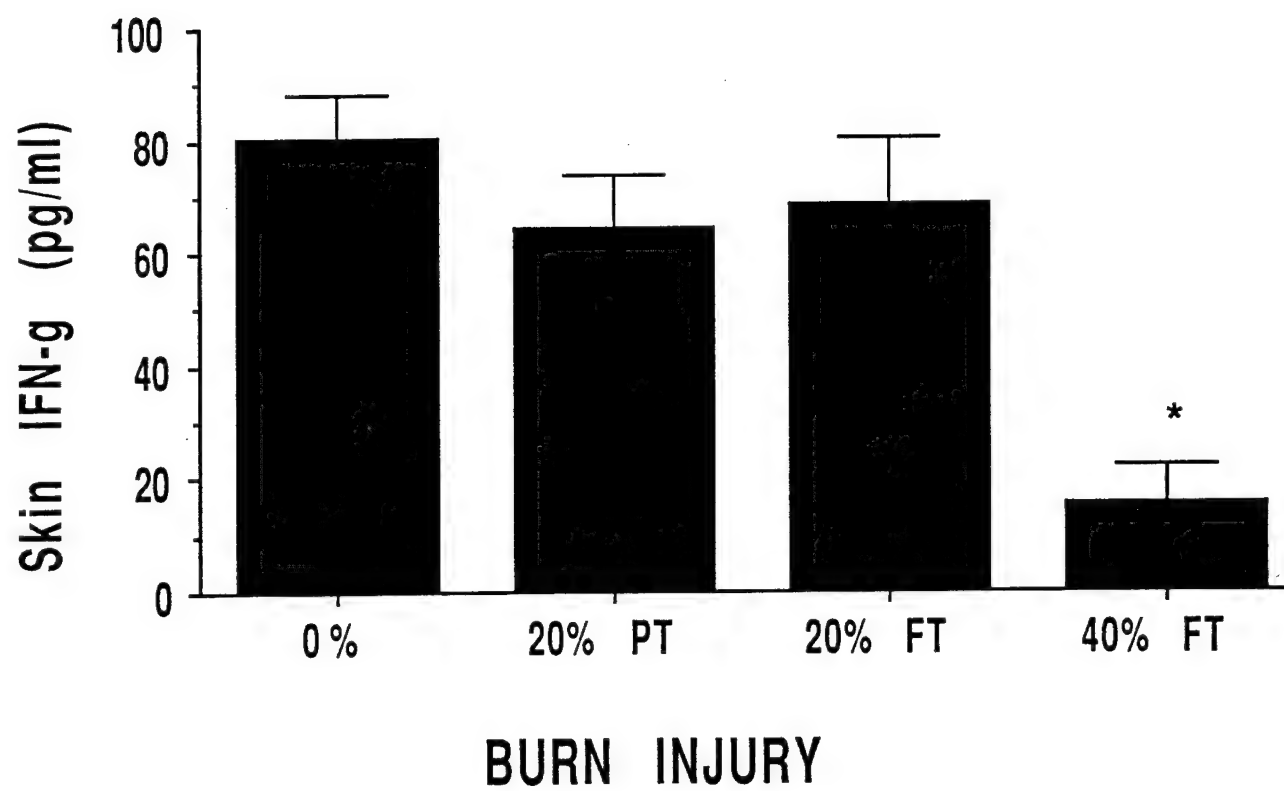


Table 1. The effect of burn size on alloantigen expression and IFN- γ levels in allografted wounds, serum, and unburned skin.

| MHC Class II | | IFN- γ Levels | | |
|--------------|--------------------------|----------------------|------------------|-----------------|
| BURN | Alloantigen Expression | (pg/ml \pm SEM) | | |
| SIZE | (relative pixel density) | WOUND | SERUM | SKIN |
| 0% | 2.81 \pm 0.34 | 167.4 \pm 36.0 | 17.9 \pm 8.8 | 80.4 \pm 8.0 |
| 20% PT | 2.49 \pm 0.38 | 409.3 \pm 69.5 | 36.1 \pm 12.8 | 64.1 \pm 9.5 |
| 20% FT | 1.54 \pm 0.25 | 322.6 \pm 35.4 | 34.8 \pm 5.5 | 68.5 \pm 11.7 |
| 40% FT | 0.47 \pm 0.08 | 47.7 \pm 10.9 | 117.0 \pm 16.0 | 15.2 \pm 6.9 |

**The Effect of Burn Injury on Allograft Rejection,
Alloantigen Processing, and Cytotoxic T Lymphocyte Sensitization**

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cytotoxic T lymphocyte, immunosuppression, cell mediated immunity

ABSTRACT

Burn injury impairs cellular immunity, increases the risk of viral infection, and delays allograft rejection, but little is known about its effect on antigen processing and cytotoxic T lymphocyte (CTL) function. This study examines the effect of burn injury on alloantigen sensitization, using an in vivo model of second set rejection and in vitro assays of CTL alloreactivity. **Methods:** Anesthetized CBA mice (n=95) received a 0%, 20%, or 40% full-thickness contact burn that was partially excised three days later and covered with autograft or C57BL/6 allograft. Two weeks postburn, mice were challenged with second set tail allografts, which were observed for rejection. Median graft survival times were compared by Wilcoxon rank and χ -square. Additional CBA mice (n=24) underwent similar burn injury, excision, and grafting. Splenocytes were harvested two weeks later and used as CTL effectors against radiolabeled targets. Dilution curves of target lysis were compared by ANOVA. **Results:** Forty-percent burn injury prolonged unprimed allograft survival from 13 to 15 days ($p<0.01$) but had a greater effect on primed allograft survival, which increased from 9 to 12.5 days ($p<0.01$). Furthermore, a 40% burn eliminated the influence of priming, resulting in second set graft survival similar to that of unburned, unprimed controls (12.5 vs 13 days, NS). While 20% burn injury did not inhibit CTL priming, a 40% burn profoundly impaired CTL function ($p<0.001$), which recovered only after six days of in vitro allostimulation. **Conclusions:** Burn injury inhibits both alloantigen priming and the immunologic memory of CTLs as a function of burn size. This impairment in alloantigen processing helps to explain defects in cellular immunity and suggests a mechanism for prolonged allograft survival and decreased viral resistance postburn.

INTRODUCTION

Through mechanisms not completely described or understood, burn injury produces defects in cellular immunity which ultimately result in host immunosuppression. Clinically, burn injury increases the risk of viral infection,¹ inhibits contact hypersensitivity,^{2,4} and delays allograft rejection,⁵⁻⁸ suggesting an impairment in antigen recognition and/or elimination. While burn injury has been shown to inhibit lymphocyte effector function⁹⁻¹³ and to alter lymphocyte cytokine production,¹⁴⁻¹⁷ little is known about the effect of burn injury on MHC-restricted antigen processing, which involves the recruitment and activation of cytotoxic T lymphocytes (CTLs).

The ramifications of impaired CTL activity include allograft nonresponsiveness and decreased host resistance to opportunistic infections.^{18,19} Although most postburn infections are bacterial in origin, the incidence and significance of viral infections may be under-appreciated. Pediatric patients with major thermal injury appear to be at increased risk for cytomegalovirus infection, which manifests itself as hepatitis and persistent fever.¹ Another consequence of postburn immunosuppression is prolonged allograft survival⁵⁻⁸ which may also be related to defects in antigen processing and CTL function. The prolonged survival of cadaveric, full-thickness skin improves patient outcome by permitting more aggressive wound excision and enabling complete wound closure. However, such functional wound coverage is only temporary, as allogeneic epidermal cells are eventually rejected upon restoration of host immunocompetence. Although not

yet achieved, the induction of permanent allograft tolerance remains a challenging but desirable goal in burn wound management.

The purpose of this study was to characterize the effect of burn injury on alloantigen processing. Specifically, we were interested in studying how thermal injury affects alloantigen sensitization. First described by Medawar in 1943 as second set rejection,²⁰ sensitization (also known as priming) involves a phenomenon in which animals previously exposed to a specific antigen will mount a more vigorous rejection response when re-exposed to that antigen. Using an *in vivo* model of second set rejection, we investigated the effect of burn injury on naive and primed allograft rejection. Additionally, *in vitro* CTL assays were used to elucidate the influence of burn injury on MHC-restricted, target specific cytotoxicity. Together, the results of these studies suggest that burn injury inhibits antigen processing as a function of burn size, and that primed allograft rejection is impaired to a greater extent than naive rejection.

MATERIALS AND METHODS

Experimental Design. To determine the effect of burn injury on alloantigen processing, we used an *in vivo* model of second set rejection and *in vitro* assays of CTL function to study the influence of burn size on alloantigen sensitization. In the first series of experiments, 95 CBA mice were randomized to receive a 0%, 20%, or 40% total body surface area (TBSA) burn, which was partially excised three days later and covered with either CBA autograft (n=45) or C57BL/6 allograft (n=50). Two weeks after grafting, the animals were then challenged with second set tail allografts, which were observed for rejection. In the second series of experiments, 24 CBA

mice received a 0%, 20%, or 40% TBSA burn, which was partially excised three days later and covered with C57BL/6 allograft. Two weeks after priming, splenocytes were harvested, stimulated with alloantigen, and used in subsequent CTL assays. Splenocytes were also collected from a fourth group of unburned, autografted CBA mice. This group served as a negative control, representing the baseline activity of unprimed CTLs against allogeneic targets.

Animal Protocols. Female CBA/J mice (4-6 week old, 15-20 grams) (H-2^k) (Harlan Sprague Dawley, Inc., Indianapolis, IN) were used as graft recipients and autograft donors in both experiments. Allogeneic splenocytes needed as stimulators in the CTL assays were obtained from age/weight/gender-matched C57BL/6 mice (H-2^b) (Charles Rivers Laboratories, Wilmington, MA), which were also used as allograft donors in both experiments. Animal protocols conformed to NIH guidelines and were approved by the UNC Committee on Animal Research.

Burn Injury Model. After induction of general anesthesia with methoxyflurane (Pitman-Moore, Washington Crossing, NJ), circumferentially clipped mice received a full-thickness flank/back contact burn, via a 10 second application of a 65g brass rod, previously heated to 100°C. Animals were then resuscitated with intraperitoneal lactated Ringer's solution (0.1 ml/g body weight), given subcutaneous morphine sulfate (3µg/g body weight), and returned to individual cages to feed ad libitum.

Concerning the contact burn, one application represents 10% of the animal's TBSA, with four separate applications necessary to create a 40% TBSA burn. Sham animals receiving the 0% TBSA burn had all of the above interventions, with the

exception of the rod application. The contact burn produces a wound of consistent depth and border, which allows for precise excision and grafting. Predictable mortality rates in unexcised mice include 0% for the sham burn, 10% for the 20% TBSA burn, and 40% for the 40% burn. In this burn model, wound infection rarely occurs, and mortality is typically due to generalized sepsis, which occurs 3-10 days after injury. Autopsy reveals intestinal dilatation and edema, consistent with ileus and partial obstruction.

Wound Excision and Primary Flank Grafting. Three days after burn injury, flank wounds were partially excised, leaving a significant amount of eschar intact to maintain host immunosuppression. Full-thickness autografts or allografts, previously debrided of adipose and washed in phosphate-buffered saline, were placed directly onto the exposed musculoskeletal fascia, were secured with skin staples, and were covered with a circumferential adhesive bandage, which was removed after one week. This sequence of burn injury, excision, and grafting was used to approximate the clinical practice of early excision and grafting.

Second Set Tail Grafting. To determine the effect of thermal injury on host sensitization, mice were challenged with alloantigen two weeks after burn injury, excision, and grafting. Anesthetized mice underwent second set tail grafting, via a previously described method,²¹ in which a 10 mm full-thickness skin allograft (C57BL/6) was placed on the dorsal tail surface and compared to distally placed 10 mm full-thickness autograft (CBA), serving as an internal control. Tail grafts were protected from mechanical disruption and organic debris by specifically designed cylindrical glass tubes, which were removed three days later. Two independent

observers assessed tail graft viability daily. Evidence for rejection was based on the objective criteria of graft color, scale integrity, and hair orientation. Median survival time (MST) of second set tail allografts was compared between the six groups and assessed for statistical significance.

CTL Alloreactivity. To determine the effect of burn injury on MHC-restricted CTL reactivity and sensitization, we studied CTL function in CBA mice receiving burn injury, partial wound excision, and flank allografting. Two weeks after sensitization with alloantigen, splenocytes were harvested, washed in culture media (RPMI 1640 media with 10% fetal bovine serum, 0.1% penicillin/streptomycin, and 5×10^{-5} M 2-ME, all obtained from the Lineberger Cancer Center, Chapel Hill, NC), and used in subsequent CTL assays. Graft recipient splenocytes (2×10^6 cells/ml, CBA mice, H-2^k) were co-cultured with growth-arrested (2000 rads) donor splenocytes (2×10^6 cells/ml, C57BL/6, H-2^b) in standard incubator conditions of 37°C and 5% CO₂.

Following 0-7 days of in vitro stimulation, CTL alloreactivity was determined by collecting effector lymphocytes and testing them on both allogeneic positive targets (EL-4 murine lymphoma cells, H-2^b, ATCC TIB 39) and MHC-identical negative targets (LTK⁻, H-2^k, ATCC CCL 1.3). After having been preloaded with 100 μ Ci of Na₂⁵¹CrO₄ (ICN Biomedicals Inc., Irvine, CA) for 30 minutes, targets were placed in round-bottom 96 well plates (1×10^6 cells/well) and mixed with CTL effectors at ratios of 1:12.5, 1:25, 1:50, 1:100, and 1:150. After a four hour, 37°C incubation in 150 μ l of RPMI 1640 with 5% FBS, target lysis was determined by measuring the release of Na₂⁵¹CrO₄ into the media. Collected supernatants were assessed for radioactivity (expressed as mean cpm) by a γ -counter. Each condition

was tested in duplicate. Specific target killing was determined by the following formula: % specific lysis = $[(\text{cpm}_{\text{sample}} - \text{cpm}_{\text{spontaneous}}) / (\text{cpm}_{\text{maximum}} - \text{cpm}_{\text{spontaneous}})] \times 100$, where $\text{cpm}_{\text{spontaneous}}$ represents spontaneous target release and $\text{cpm}_{\text{maximum}}$ represents the radioactivity of targets lysed by 5% Triton X-100. Average spontaneous target release was less than 10% of the potential maximum release. A total of 12 assays were performed, after 0, 3, 4, 5, 6, and 7 days of stimulation, against positive and negative targets. This experiment was repeated and yielded similar results.

Statistics. Median survival times (MST) of second set tail allografts were compared between groups by Wilcoxon rank and χ -square analysis. CTL alloreactivity, as signified by % specific lysis, was compared between groups by two-way ANOVA with replication. Statistical significance was defined for differences where $p < 0.05$.

RESULTS

Second Set Rejection. To characterize the effect of thermal injury on alloantigen processing, we used a model of second set rejection, in which previously burned mice underwent wound excision and flank grafting, followed two weeks later by secondary tail allografting. We hypothesized that prior exposure to alloantigen would result in a more vigorous rejection response when mice were challenged with second set allografts. Furthermore, we hypothesized that burn injury would impair both primed and unprimed rejection of tail allografts, as evidenced by prolonged survival of the second set tail grafts.

Median tail allograft survival times, along with range and group size, are listed in Table 1. Survival curves, depicting graft survival as a function of time, can

be found in Figure 1. As expected, unburned mice primed with alloantigen rejected second set allografts more rapidly than did the unburned, autografted group (9 vs. 13 days, respectively; $p < 0.001$). Burn injury increased tail allograft survival in both primed and unprimed groups. While burn injury prolonged unprimed graft survival in the 20% TBSA burn group (from 13 to 14 days, $p < 0.01$) and the 40% burn group (from 13 to 15 days, $p < 0.001$), the difference between these two groups was not significant. Concerning the primed group, a 20% burn increased tail allograft survival from 9 to 10 days ($p < 0.05$), and a 40% burn prolonged allograft survival from 9 to 12.5 days ($p < 0.001$). The difference between these primed groups (20% vs 40% burn) was statistically significant ($p < 0.01$). Although burn injury impaired allograft rejection in both the primed and unprimed groups, burn injury had a greater effect in the primed group, suggesting a specific defect in alloantigen sensitization. Furthermore, a 40% burn eliminated the effect of priming, when compared to allograft rejection in unprimed, unburned mice (12.5 vs 13 days, NS).

CTL Alloreactivity. After using this in vivo model of rejection to study the effect of burn injury on antigen processing, we then asked whether or not defects in CTL activity might account for this observation of impaired alloantigen priming. Splenocytes from allografted mice who had received a 0%, 20%, or 40% burn, as well as from autografted mice who had received a 0% burn, were co-cultured with growth-arrested allogeneic stimulators and used as effectors in subsequent CTL assays. Dilution curves representing specific lysis of positive and negative targets are depicted in Figures 2-5.

CTL alloreactivity was not observed until the third day of in vitro stimulation. Unburned, primed mice displayed the greatest CTL activity against positive targets, along with primed mice receiving a 20% TBSA burn injury (Figure 2, $p < 0.001$). CTLs from unburned, unprimed mice, as well as CTLs from primed mice with the 40% burn, demonstrated no significant activity, compared to the nonspecific lysis of negative targets. After four days of in vitro stimulation, CTL activity improved in all of the primed groups, but profound suppression of CTL activity by a 40% burn was still observed (Figure 3, $p < 0.001$). While the 20% primed group had slightly decreased CTL function compared to the 0% primed group, the 40% primed group demonstrated insignificantly improved CTL function compared to the unprimed, unburned group.

CTL activity in the unburned, unprimed control group improved dramatically after five days of stimulation (Figure 4). However, depressed CTL alloreactivity in the 40% burn group persisted and did not fully recover until the final day of the assay (Figure 5). After six days of simulation, primed CTLs from all three burn groups demonstrated greater activity than unprimed CTLs, except at an E:T ratio of 1:100. This complete recovery of impaired CTL function postburn suggests that defects in antigen priming, while significant, are not permanent and can be reversed by in vitro stimulation with alloantigen.

DISCUSSION

In this series of experiments, we provide evidence that thermal injury produces identifiable defects in alloantigen processing. Specifically, burn injury inhibits both primed and unprimed allograft rejection, with the degree of immune

dysfunction dependent on the size of the burn wound. Furthermore, alloantigen sensitization appears to be more impaired than naive alloantigen elimination, in our model of second set rejection. These *in vivo* findings correlate with *in vitro* studies of CTL alloreactivity after burn injury. While a 20% TBSA burn had minimal effect on primed CTL target cytotoxicity, a 40% burn dramatically mitigated CTL sensitization. Although 40% burn injury negated the immunologic memory of CTLs, target-specific cytotoxicity fully recovered, but only after six days of *in vitro* stimulation with alloantigen.

Immunosuppression after thermal injury has been extensively studied, but the full range of defects in cellular immunity has yet to be described. Assays of cellular immune function postburn reveal a decreased lymphocyte proliferation in response to mitogens,^{14,22} decreased lymphocyte production of IL-2,^{14,15} and decreased lymphocyte effector function.⁹⁻¹³ Furthermore, fluorescence-activated cell sorting (FACS) demonstrates decreased numbers of T helper and T cytotoxic lymphocytes, as well as the emergence of suppressor lymphocytes, in both human and animal models of burn injury.²³⁻²⁵

This disruption of cellular immunity has significant clinical consequences, increasing the risk for infectious complications, which remain the leading cause of post-resuscitation deaths following burn injury.²⁶ While gram negative sepsis accounts for the majority of postburn infections,²⁷ the incidence of viral, fungal, and other opportunistic infections are occurring with increasing frequency and may not be fully appreciated.²⁸ In a prospective case series, Linnemann and MacMillan reported that CMV infection occurred in 33% of pediatric burn patients and HSV

infection occurred in 25%.¹ Additionally, those patients with larger burns were more likely to develop antiviral antibodies and have positive viral cultures. This susceptibility to viral infection suggests that burn injury impairs the effector function of CTLs, the component of cellular immunity responsible MHC-directed elimination or clearance of viral antigen.

Impaired antigen processing postburn is implicated by defects in contact hypersensitivity, which is a nonspecific but quantitative indicator of cell mediated immunity. Hansbrough et al have shown that a 20% burn injury can significantly reduce contact hypersensitivity, as measured by ear swelling, in animals that were sensitized with and re-exposed to dinitrofluorobenzene.^{2,3} Early wound excision restores contact hypersensitivity in the burned host,² while transfer of the burn eschar to unburned hosts also transfers these defects in antigen processing.³

Other evidence for impaired antigen processing postburn comes from the observation that burn injury mitigates the host-vs-graft response. Cetinkale et al noted that burn injury decreases the alloreactive response when a host is challenged with alloantigen, as measured by the popliteal lymph node assay.⁴ Furthermore, prolonged allograft survival postburn is well-documented⁵⁻⁸ and has been utilized to permit early wound excision of the massively burned patient. Markley et al reported that burn injury, as well as septic and hyperosmolar shock, produced considerable immune dysfunction such that allogeneic skin grafted three weeks after injury had prolonged survival.⁶ Additionally, second set allografts placed 70 days after burn injury also had prolonged survival, suggesting a long-lasting defect in alloantigen sensitization. Although our experimental model involved much

earlier excision and grafting, these results are consistent with our findings that burn injury impairs primed and unprimed allograft rejection.

The mechanism for delayed allograft rejection may be related to postburn defects in CTL function, which is a critical effector component of allograft rejection. Previous work by Markley et al demonstrates that a 66% TBSA burn injury inhibits the effector function of CTLs from BALB/c mice presensitized with an intraperitoneal injection allogeneic EL-4 cells.⁹ This impaired sensitization was present when burn injury occurred between seven days before and 13 days after exposure to alloantigen. Furthermore, burn injury appeared to inhibit the afferent side of alloantigen sensitization, in which EL-4 cells from burned C57BL/6N donors failed to prime BALB/c hosts for target-specific CTL cytotoxicity.¹⁰ In our experiment, mice were primed instead with alloantigen from skin allografts, and we observed that a 40% burn injury was required to inhibit primed CTL alloreactivity.

Successful long-term coverage with allografts remains an elusive goal in burn wound management. Although allograft rejection can be delayed by the use of cyclosporin²⁹⁻³¹ and azathioprine,³² by allograft irradiation or treatment with glucocorticoids,³³ or after bone marrow transplantation,⁸ skin allografts used for burn wound coverage are ultimately rejected upon restoration of host immunocompetence. Understanding how burn injury affects alloantigen processing, however, may someday permit the manipulation of host and/or graft to effect the permanent survival of allografts in burn wound coverage.

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TABLES

Table 1. The effect of burn injury and priming on the survival of second set tail allografts. Mice receiving primary flank allografts are defined as PRIMED, whereas mice receiving primary flank autografts are defined as UNPRIMED. MST=median survival time of secondary tail allografts.

| | PRIMED | | | UNPRIMED | | |
|------------|--------------------------------|----------|----------|--------------------------------|----------|----------|
| | Primary flank graft: allograft | | | Primary flank graft: autograft | | |
| | 0% burn | 20% burn | 40% burn | 0% burn | 20% burn | 40% burn |
| MST, days | 9 | 10 | 12.5 | 13 | 14 | 15 |
| MST, range | 7-11 | 8-12 | 10-16 | 10-15 | 12-17 | 13-18 |
| N | 18 | 16 | 16 | 15 | 17 | 13 |

FIGURES

Figure 1. Survival curves of secondary tail allografts, depicting the effect of thermal injury and priming on second set rejection. Burn injury significantly prolonged second set tail graft survival in both the primed (Allo) and unprimed (Auto) groups ($p < 0.05$). Sensitized rejection was impaired to a greater extent than naive rejection.

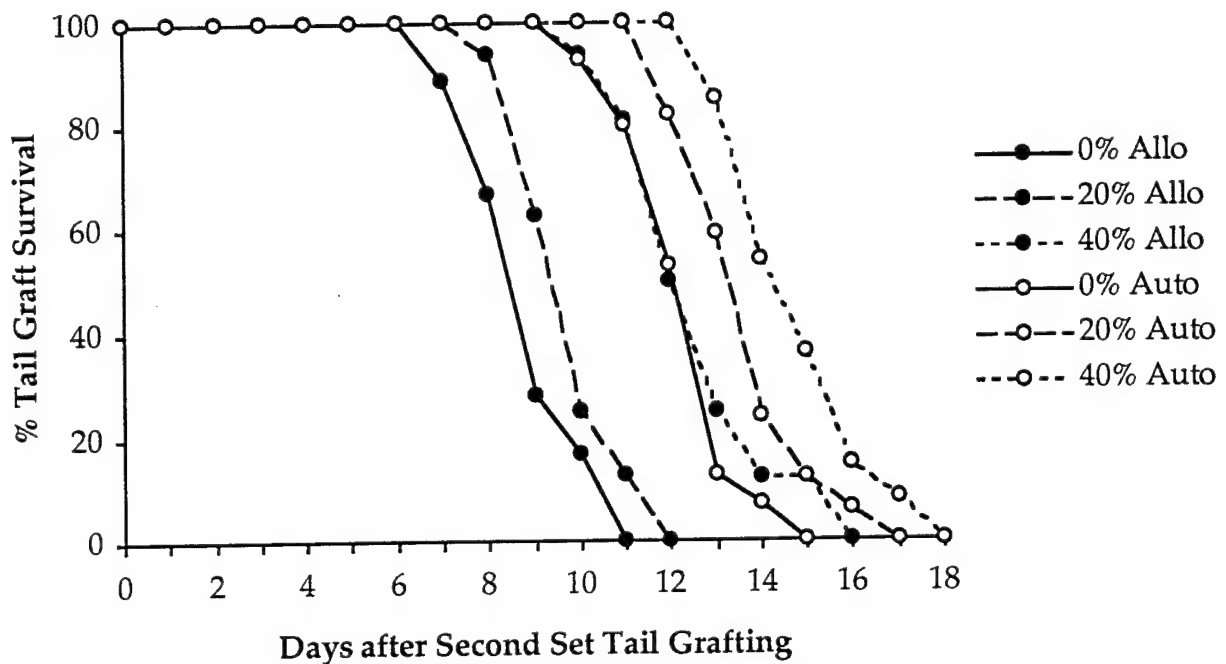


Figure 2. The effect of burn injury on CTL alloreactivity, 3 days after in vitro stimulation.

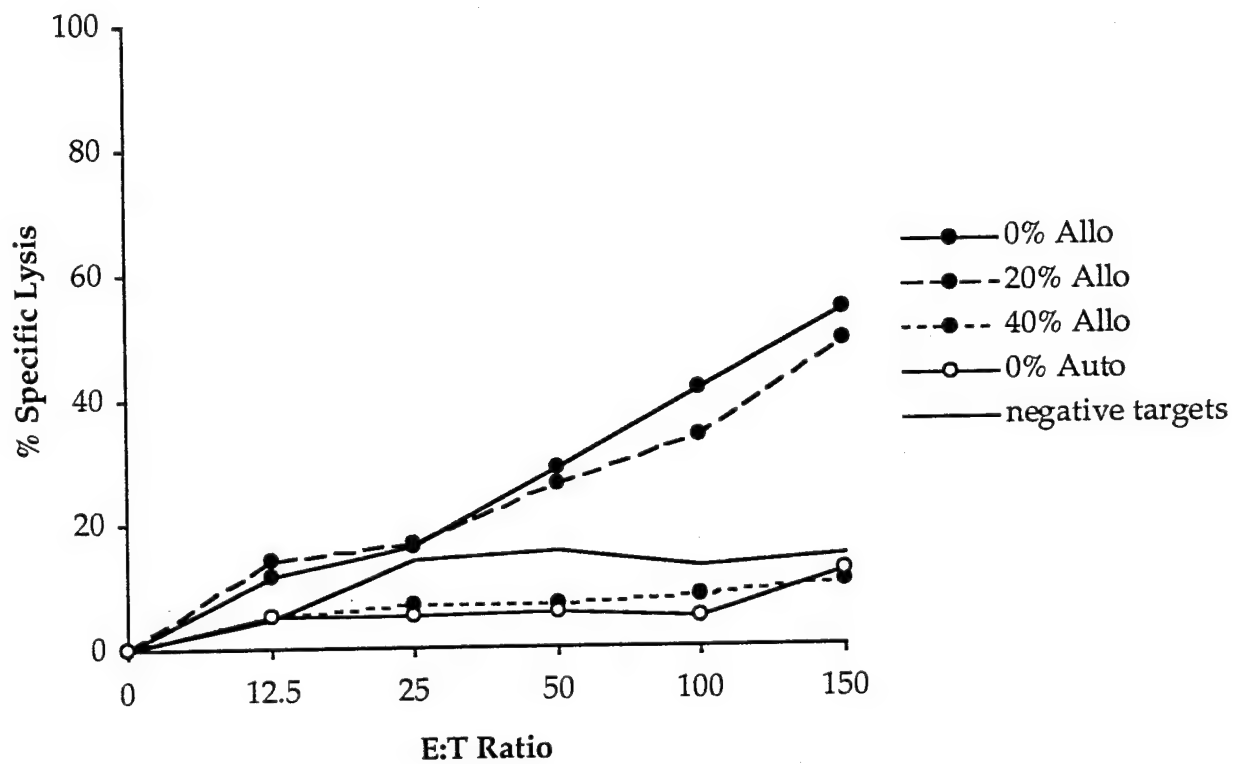


Figure 3. The effect of burn injury on CTL alloreactivity, 4 days after in vitro stimulation.

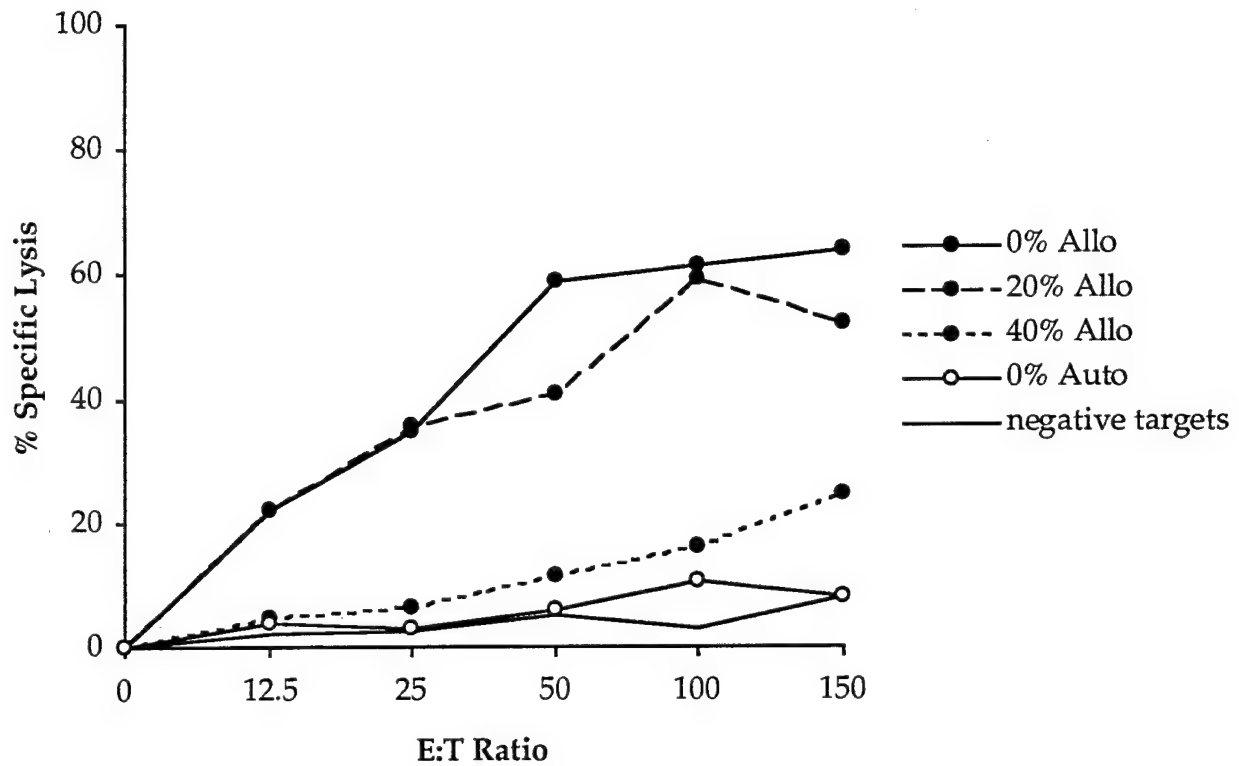


Figure 4. The effect of burn injury on CTL alloreactivity, 5 days after in vitro stimulation.

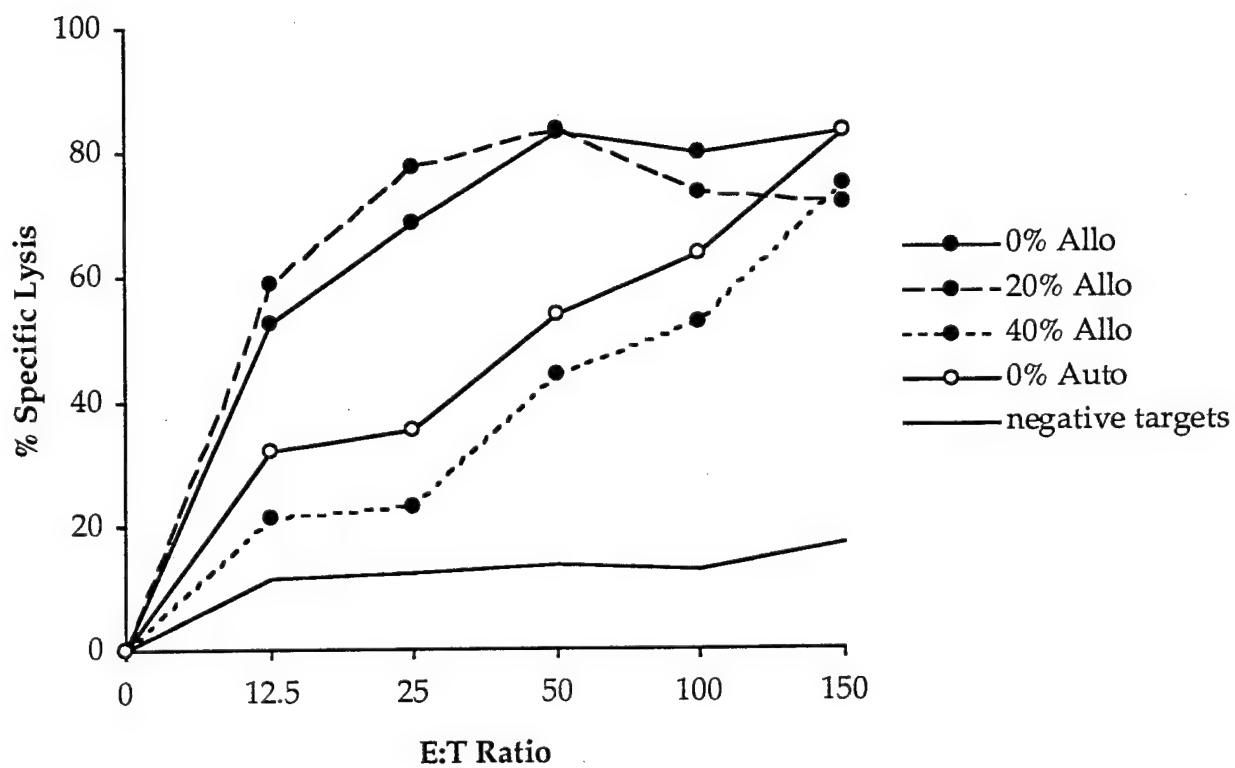


Figure 5. The effect of burn injury on CTL alloreactivity, 6 days after in vitro stimulation.

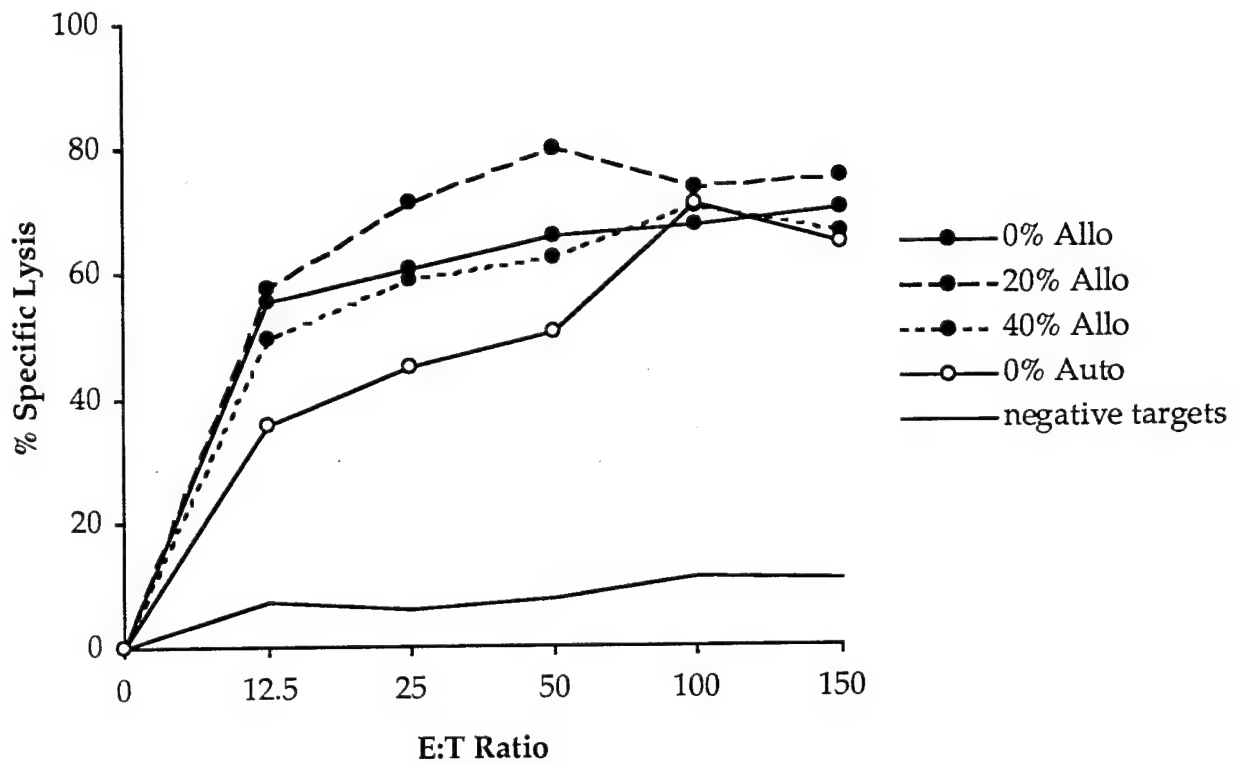


FIGURE LEGENDS

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**THE EFFECT OF THERMAL INJURY ON LOCAL WOUND PRODUCTION OF
INTERFERON- γ , TUMOR NECROSIS FACTOR- α , AND INTERLEUKIN-10**

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The prolonged survival of cultured keratinocyte (CK) allografts in patients with major thermal injury can partly be explained by impaired host responsiveness^{1,2} but may also be due to decreased graft immunogenicity.³ We have recently demonstrated that increasing burn size progressively down-regulates expression of keratinocyte Class II histocompatibility antigens, which are critically involved in allograft recognition and rejection. However, the mechanisms concerning keratinocyte alloantigen expression have not yet been fully described. The purpose of this experiment was to characterize the effect of burn injury on local wound production of interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), and interleukin-10 (IL-10), three cytokines implicated in the regulation of Class II alloantigens.⁴⁻⁶

MATERIALS AND METHODS

Sixty-nine CBA mice (H-2^k) received a 0%, 20% partial thickness (PT), 20% full-thickness (FT), or 40% FT contact burn. All mice except the control group received methoxyflurane anesthesia, clipping, lactated Ringer's resuscitation, and morphine sulfate for post-burn pain control. Wounds were partially excised 48 hours later and grafted with CK allografts from C57BL/6 donors (H-2^b). CK allografts had previously been grown to confluence by co-culturing allogeneic donor keratinocytes with a growth-arrested feeder layer of LTK⁻ fibroblasts (H-2^k), syngeneic with graft recipients. Five days after burn injury, local production of IFN- γ , TNF- α , and IL-10 was determined. Allograft wound biopsies were sonicated and supernatants frozen at -80°C. Cytokine levels were measured in duplicate by ELISA and compared by Fisher's ANOVA.

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RESULTS

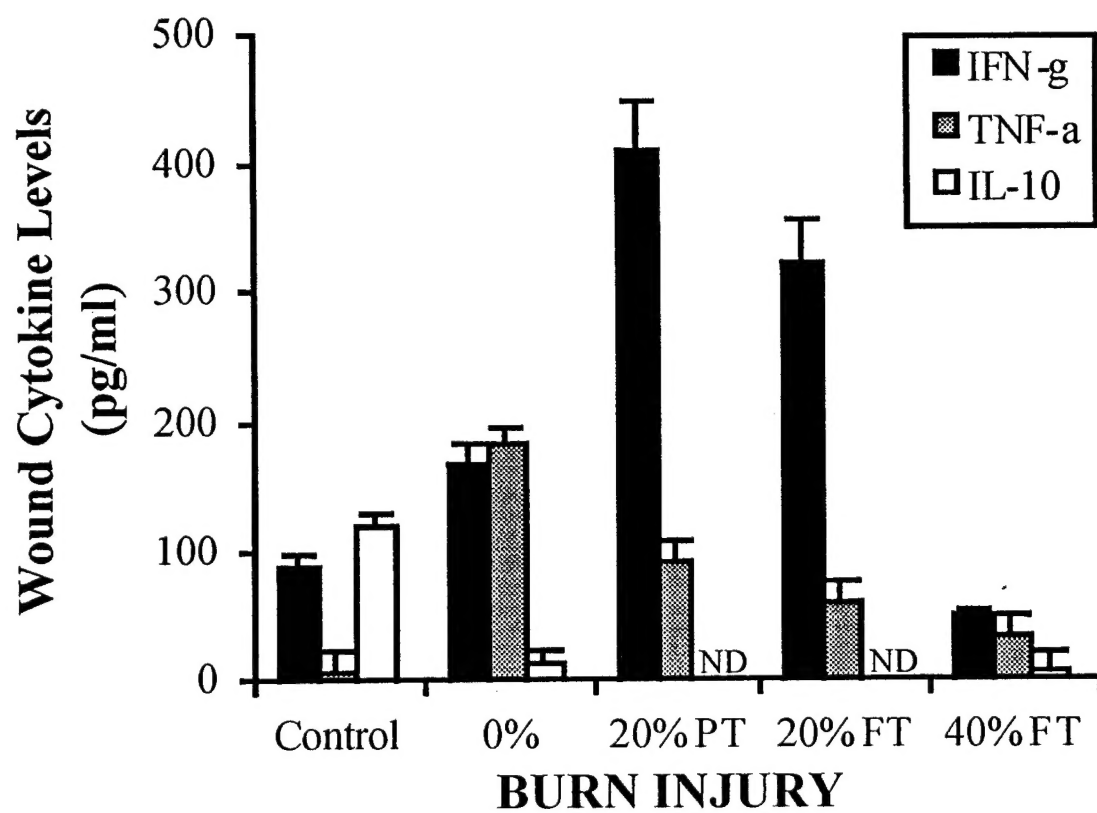
Allograft levels of IL-10 were profoundly decreased after burn injury, compared to the control group ($p < 0.01$, Figure 1). IFN- γ levels significantly increased after 20% burn injury but decreased after 40% burn injury ($p < 0.05$). TNF- α levels increased dramatically after minimal intervention (0% burn injury) but decreased as burn size increased ($p < 0.05$).

CONCLUSIONS

Local wound production of IFN- γ , TNF- α , and IL-10 is dependent on both the depth and size of the burn wound. Impaired alloantigen expression after 40% burn injury can be explained by decreased IFN- γ , but diminished alloantigen expression after 20% burn injury (which augments wound IFN- γ) may be due to the opposing effects of increased TNF- α . Such variation in IFN- γ and TNF- α levels post-burn may be related to depressed tissue levels of IL-10, which is required for normal cytokine regulation.

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FIGURE

LEGEND

Figure 1. The effect of burn injury on wound levels of IFN- γ , TNF- α , and IL-10. Forty-eight hours after receiving control, 0%, 20% PT, 20% FT, or 40% FT TBSA contact burns, CBA mice (n=69) underwent partial wound excision and were grafted with cultured, allogeneic keratinocytes from C57BL/6 donors. Three days later (five days after burn injury), allograft biopsies were obtained and assayed for IFN- γ , TNF- α , and IL-10 by ELISA. Cytokine levels are expressed as pg/ml. Abbreviations: IFN- γ , interferon- γ ; TNF- α , tumor necrosis factor- α ; IL-10, interleukin-10; PT, partial thickness; FT, full-thickness; TBSA, total body surface area; ND, not detected.

ALLOGENEIC FIBROBLASTS USED TO GROW CULTURED EPIDERMAL AUTOGRAFTS PERSIST IN VIVO AND SENSITIZE THE GRAFT RECIPIENT FOR ACCELERATED SECOND SET REJECTION

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Introduction: Cultured epidermal autografts (CEAs) have been used for wound coverage in patients with massive burns and other skin defects. However, CEAs often display late breakdown, which may be immunologically mediated and initiated by persistent foreign fibroblasts used as a feeder layer to optimize keratinocyte growth. This study investigates if these fibroblasts, previously shown to persist in vitro, survive after grafting and induce host sensitization to alloantigen.

Methods: CEAs from CBA donors (H-2^k) were grown on allogeneic NIH 3T3 (H-2^d) or syngeneic LTK (H-2^k) fibroblasts (FB), which were removed by trypsinization 7 days later. CBA mice (n=54) were flank grafted with NIH allografts (positive control), CEA/3T3s, CEA/LTKs, or CBA autografts (negative control). Hosts were challenged with second set NIH tail allografts 3 weeks later. Median graft survival was compared between groups by Wilcoxon rank and χ^2 . Additional CBA mice (n=15) received CEAs that were biopsied 0, 4, and 8 days after grafting. The presence of allogeneic fibroblasts was determined by Western immunoblotting, using KL295, a monoclonal antibody which recognizes H-2^d (but not H-2^k) Class II histocompatibility antigens.

Results: Allogeneic fibroblasts persisted after grafting but decreased over time, as determined by alloantigen expression on Western immunoblots. Accelerated tail graft rejection occurred in hosts primed by NIH allografts (*p<0.05), as well as by CEAs grown with an allogeneic (3T3) but not syngeneic (LTK) feeder layer (*p<0.05).

| Primary Flank Graft | NIH allo (+) control | CEA/3T3 allogeneic FB | CEA/LTK syngeneic FB | CBA auto (-) control |
|--------------------------|-------------------------|--------------------------|-------------------------|-------------------------|
| Secondary Graft Survival | 9 days* | 10 days* | 12 days | 12 days |

Conclusions: Immunogenic fibroblasts used to grow CEAs survive in vivo and sensitize the graft recipient for accelerated second set rejection. These persistent cells initiate an inflammatory response which may result in late graft breakdown and may limit the utility of CEAs grown with a foreign fibroblast feeder layer.

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